Identification and characterization of secreted proteins of *Theileria lestoquardi* schizonts

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Abstract

T. lestoquardi is a tick-borne parasite that infects leukocytes of sheep and goats. It causes the death of millions of sheep and goats every year and continues to spread in the world. The disease is mainly caused by the schizont stage, which induces transformation of the infected cell to a state of uncontrolled proliferation. In response to the proliferation of infected cells, there is evidence that the host deploys its immune system to eliminate the pathogen by cytotoxic T lymphocyte (CTL) responses. The purpose of this study was to identify schizont secreted proteins, which are likely to include molecules targeted by the immune response as well as those associated with the transformation process. An efficient method for the lysis and purification of schizonts from infected cells was established. This involved use of complement treatment to release schizonts from infected cells and an optimal density of Nycoprep gradient medium to separate schizonts from host cell components. An optimised method for metabolic labelling of schizont-infected cells was also established. Putative secreted proteins were detected in metabolically labelled schizont culture supernatants by SDS-PAGE and autoradiographic analysis. Using two-dimensional electrophoresis, mass spectrometry, including MALDI-TOF and Q-TOF, and N-terminal sequencing analysis, three putative secreted proteins of parasite origin were identified, namely *Theileria* mhsp70, hsp70 and inorganic pyrophosphatase. Seven host-derived proteins were also identified, namely mhsp70, hsp60, mitochondrial ribosomal protein L12 (MRPL12, TLS4), ATP synthase-δ, ATP synthase 6, PDH-E1β and ATP-dependent proteinase SP-22. Further investigation of the relationship of the host-derived proteins with the schizont using dual-labelling and confocal microscopy analysis provided evidence that the host-derived protein, MRPL12, is associated with intracellular schizonts with some co-localising with schizont mitochondria. The analysis also revealed that some host mitochondria are closely associated with the schizonts of *T. lestoquardi*.

Declaration

The work presented in this thesis was carried out at the Moredun Research Institute, Edinburgh. The experimental work and the interpretation of the results were undertaken by the author. Contributions to the work in this thesis by colleagues are fully acknowledged in the text.

This work has not been nor is currently being submitted in any form to any university for the award of a degree.

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Abbreviations

2DE	Two-dimensional electrophoresis
aa	Amino acid
BFA	Brefeldin A
CHAPS	3-(3-cholamidopropyl-dimethylammonio)-1-propane sulfonate
CHCA	α-cyano-4-hydrocycinnamic acid
CID	collision-induced dissociation
CK2	Casein kinase II
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EM	Electron microscopy
FCS	foetal calf serum
GUTS	Ground up tick supernatant
HBSS	Hanks' buffered saline solution
HPLC	High-performance liquid chromatography
hrs	hour(s)
HRP	Horse radish peroxidase
Hsps	Heat shock proteins
IDA	Information Dependent Acquisition
IEF	Isoelectric focusing
IFA	Immunofluroscence assay
IMDM	Iscove's Modified Dulbecco's Medium
IPG	Immobilized pH gradient
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MCF-DMEM	methionine/cysteine-free Dulbecco's Modified Eagle's medium
MHC	major histocompatibility complex
min	minute(s)
MRI	Moredun Research Institute
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass/charge ratio

NF-κB	Nuclear factor kappa B
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pI	Isoelectric point
PI	propidium iodiol
PIM	polymorphic immunodominant molecule of *T. parva*
PITC	Phenylisothiocyanate
PMF	Peptide-mass fingerprinting
ppm	parts per million
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene difluoride
QAE	Quarternary aminoethyl
Q-TOF	Quadrupole-time of flight
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SLAG-1	*T. lestoquardi* sporozoite antigen 1
srRNA	small-subunit ribosome RNA
te	tick equivalent
TLL	*T. lestoquardi*-infected leukocytes
TLS	*T. lestoquardi* schizonts

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Chapter 1: General introduction

1.1 *Theileria* parasites

Theileria parasites are members of the phylum *Apicomplexa* which are obligate intracellular protozoan parasites that, in general, have complex life cycles. The *Apicomplexa* includes several species of both medical and veterinary importance, such as *Plasmodium, Toxoplasma, Babesia* and *Theileria.*

Theileria species are tick-borne parasites that mainly infect cattle, sheep and goats. Brown (1983) classified them as follows:

Sub-kingdom	*Protozoa*
Phylum	*Apicomplexa*
Class	*Sporozoea*
Subclass	*Piroplasmidae*
Family	*Theileriidae*
Genus	*Theileria*

The most pathogenic *Theileria* species are *T. parva*, *T. annulata* and *T. lestoquardi* (Morel 1981). They are characterized by having an intracellular schizont stage that multiplies in leukocytes. *Theileria* species are highly adapted to invade and survive within a range of different cells in their tick and mammalian hosts (Brown 1983).

1.1.1 *T. parva* and *T. annulata* species

T. parva parasites were first described in German East Africa by Koch (1898) and had become the most devastating protozoan parasites of cattle in Southern Africa at the turn of the 20th century (Henning 1956). At the same time as *T. parva* infection was spreading in Rhodesia and South Africa, a similar disease manifested itself in Transcanlasia (Dschunkowsky et al. 1904). This proved to be Tropical theileriosis or Mediterranean Coast Fever, caused by *T. annulata* species. The genus was named as Theileria in the biography of Sir Arnold Theileria in 1906 (Henning 1956). Little was known of the life cycle of the parasites in the tick vector when Cowdry and Ham, using

classical histological techniques, defined the life cycle of *T. parva* parasite in its vector *Rhipicephalus appendiculatus* (Cowdry et al. 1932).

T. parva infects cattle and buffalo and its main tick vector is *R. appendiculatus*. *T. parva* infection occurs in eastern, central and southern Africa and it is highly pathogenic to cattle and buffalo, causing Corridor disease, East Coast Fever and Rhodesian malignant theileriosis (Uilenberg et al. 1976). *T. annulata* species also infects buffalo and cattle. It causes mild disease in buffalo but is highly pathogenic to cattle, especially imported breeds, causing tropical theileriosis. The main vectors of *T. annulata* in the field are *Hyalomma anatolicum anatolicum*, *H. detritum* and *H. asiaticum*. *T. annulata* infection occurs mainly in northern Africa, southern Europe, the Middle East and central Asia (Brown 1990).

1.1.2 Economic importance

T. annulata and T. parva species account for nearly all the cases of bovine theileriosis in large areas of the tropical and sub-tropical world. Robinson (1982) estimated that 250 million cattle were at risk from *T. annulata* infection in North Africa, southern Europe, the Middle East and central Asia. In India, the potential economic losses have been estimated to be $ 800 million per annum, or 10 % of the gross national product (Brown 1990). Twenty million cattle were estimated to be at risk from *T. parva* infection in eastern, central and southern Africa (Irvin 1987) and the economic losses attributable to infection are believed to exceed $ 168 million per annum, which includes an estimated mortality of 1.1 million cattle (Mukhebi et al. 1992).

In addition to *T. parva* and *T. annulata* which mainly infect cattle and buffola, another *Theileria* species, *T. lestoquardi,* is regarded to be highly pathogenic for sheep and goats.

1.2 *T. lestoquardi*

1.2.1 The importance of *T. lestoquardi*

Originally known as *T. hirci* (Dschunkowsky et al. 1924), *T. lestoquardi* causes ovine malignant theileriosis. The species was first reported by Mason in the 1910s and was found by Littlewood (1915) to be distinct from Babesia parasites. *T. lestoquardi* infects both sheep and goats (Baumann 1939; Dschunkowsky et al. 1924) and its main tick vectors are described as *H. a. anatolicum*, *H. marginatum* and *R. sanguineus* (Hooshmand-Rad et al. 1973; Razmi et al. 2003). *T. lestoquardi* infection occurs mainly in south-eastern Europe, northern Africa, and western and central Asia (Jianxun et al. 1997; Uilenberg 1981). Considered by Littlewood (1915) and Hooshmand-Rad (1973) to be the only species of *Theileria* parasites that is highly pathogenic to sheep and goats, it has also been reported in Syria (Alyasino et al. 1999). A serological survey study showed that, between 1994 to 1997, a mean seroprevalence of 59% was found in Syrian 913 sheep samples (Alyasino et al. 1999).

T. lestoquardi infection causes the death of millions of sheep and goats every year and is continuing to spread in the world (Uilenberg 1981). Estimates of the mortality and morbidity of the disease vary. Morbidity rates of 100% are observed in sheep introduced from malignant theileriosis-free areas into highly enzootic regions (Hooshmand-Rad et al. 1973; Yakimoff 1929). However, mortality rates in sheep have been reported to range from 16% to 89.74 % (Baumann 1939; Hooshmand-Rad et al. 1973) and 85.49 % mortality has been reported in goats (Kardassis et al. 1964). Although only 39 sheep were used in Hooshmand-Rad's study, the morbidity and mortality rate was consistent with reports of Yakimoff (1929) and Kardassis et al (1964).

1.2.2 In vitro culture of *T. lestoquardi*

Methods for the culture of *T. lestoquardi* schizonts in vitro were unavailable until Hooshmand-Rad successfully cultured the parasite in ovine lymphocytes (Hooshmand-Rad 1985). Almost 99% of lymphocytes were found to be infected when stained by Giemsa. Two *T. lestoquardi*-infected cell lines were established and one was subcultured 80 times and another 60 times. In both lines, infected cells tended to adhere

and form clumps which was similar to those described for *T. parva* and *T. annulata* parasites (Brown et al. 1978; Hulliger 1965). The success of *T. lestoquardi*-infected cell cultivation has provided a valuable system for studying the mechanisms of cell transformation and vaccine development.

1.2.3 Available vaccines for control of *T. lestoquardi* infection

An attenuated cell vaccine is available for the control of *T. lestoquardi*-infection and has been used in the field (Hawa et al. 1981). The principle behind attenuated cell line vaccines for *Theileria* parasites is that long-term culture can attenuate schizont-infected cells, so that their pathogenicity is reduced, but their infectivity is retained (Sutherland et al. 1996). Attenuated cell vaccines have been used successfully for control of *T. annulata* for over 30 years in many countries, including Israel, Iran, India, Turkey, Iraq and China (Pipano 1995).

Since tissue culture was successfully established by Hooshmand-Rad (1975), *T. lestoquardi* schizont-infected cells were attenuated by the long-term culture and used as a live vaccine in sheep and goats. After challenge with sporozoites, all control animals died of acute theileriosis, whereas the vaccinated animals did not produce the erythrocytic forms of the parasite and resisted the challenge (Hooshmand-Rad 1985). This attenuated vaccine is widely used in Iran where hundreds of thousands of sheep are immunized annually (Hawa et al. 1981).

Although the attenuated vaccine is widely used for the control of *T. lestoquardi* parasites, it has a number of disadvantages. For example, it requires a cold chain and may not protect against heterologous challenge. In addition, multiple tissue culture passages are required for attenuation and, because live parasites are used, the vaccine induces a carrier state. Hence there is a demand for an alternative vaccine, preferably based on subunit antigens of the parasite.

1.2.4 *T. lestoquardi* molecules with potential for vaccine development

1.2.4.1 SLAG-1

T. lestoquardi sporozoite lestoquardi antigen 1 (SLAG-1) is the homologue of the sporozoite surface antigens of *T. annulata* SPAG-1 (Williamson et al. 1989) and *T. parva* P67 (Musoke et al. 1992), which have been considered as candidates both for inclusion in a sub-unit vaccine and as ligands for host cell recognition (Skilton et al. 2000). Antibodies raised against these antigens have been shown to neutralise sporozoite infectivity for host cells *in vitro*. The antigens contain conserved B-cell epitopes, and show considerable sequence similarity (Boulter et al. 1995; Musoke et al. 1929).

Skilton et al. (2000) used conserved sequences of SPAG-1 and P67 genes to design primers for PCR and used them to obtain a gene designated SLAG-1 from *T. lestoquardi* parasites. The SLAG-1 gene encodes a predicted protein of 723 amino acids with a calculated molecular mass of 76258 Da. The deduced SLAG-1 protein sequence exhibited 42% and 58% amino acid identity with P67 and SPAG-1 respectively, compared to 40% identity between P67 and SPAG-1 (Skilton et al. 2000). Recombinant P67 has been used to immunize cattle and gives rise to 60-70% protection against severe disease after laboratory *T. parva* challenge (Musoke et al. 1992). Immunisation with SPAG-1 provides some protection against *T. annulata* infection and gives rise to a degree of cross protection against *T. parva* infection (Boulter et al. 1995).

Studies are in progress to express full-length recombinant SLAG-1 and to determine the extent of antigen polymorphism between different stocks of *T. lestoquardi*. Vaccination studies of SLAG-1 antigen need to be carried out *in vivo* to determine its protective effect against experiment challenge with *T. lestoquardi* sporozoites.

Immunisation of cattle with recombinant P67 or SPAG-1 affords a degree of cross-protection against *T. annulata* and *T. parva* infection, respectively (Boulter et al. 1995; Musoke et al. 1992). Therefore, it is possible to combine components of P67, SPAG-1, SLAG-1, and additional potential antigens to prime protective immune

responses against *Theileria* infections in livestock. Potentially, this would provide an effective and valuable method for controlling *Theileria* infection.

1.2.4.2 *T. lestoquardi* PIM homologue

The polymorphic immunodominant molecule (PIM) of *T. parva* is the predominant antigen recognized by antisera from immune cattle on Western blot analysis of schizont-infected lymphocytes (Toye et al. 1991). The PIM antigen is present in all *T. parva* stocks and in the related subspecies, *T. p. bovis* and *T. p. lawrencei*. It has been shown that the antigen is present both in *T. parva* sporozoite and schizont stages (Toye et al. 1991). In the schizont stage of *T. parva,* the PIM is observed localized on the schizont surface (Shapiro et al. 1987). The PIM molecule has been extensively characterized and used in recombinant form for diagnosis (Katende et al. 1998). There is evidence that the PIM molecule is secreted by microspheres of sporozoites (Skilton et al. 1998).

To develop methods and to prepare reagents suitable for parasite detection and for epidemiological studies, a polymorphic gene was identified from *T. lestoquardi* cDNA library by Schnittger et al. (2002). The predicted amino acid sequence of the gene was found to be closely related to the PIM of *T. parva,* with 36% amino acid identity. The gene is 1152 bp in length and contains an open reading frame (ORF) that encodes a protein with 312 amino acid (aa) residues and a predicted molecular weight of about 35 kDa. The deduced protein sequence contains a cleavable N-terminal secretion-signal sequence of 19 aa, predicted to target the protein via the vesicular pathway. Additionally, three transmembrane regions exist in the C-terminal end, suggesting this protein is located at the parasite membrane (Schnittger et al. 2002).

Compared to another highly pathogenic *Theileria* parasite which causes fatal disease of sheep and goats in China (called *T. china*), the gene predicted sequences revealed that the N-terminal part (25 aa) and the C-terminal part (181 aa) are highly conserved (93%), while there is low identity between the central region (131 aa). The central region differs from constant regions by a very high composition of negatively charged amino acids, since asparagine and glutamine account for 28% of the amino acid

residues (Schnittger et al. 2002). The vaccine potential of *T. lestoquardi* PIM homologue has not been investigated.

1.2.5 Phenotype of *T. lestoquardi*-transformed cells

To determine which cell phenotypes are infected by *T. lestoquardi* sporozoites, Hooshmand-Rad et al. (1993) described some features of ovine lymphoid cells infected by *T. lestoquardi* parasites. They found that *T. lestoquardi* -infected sheep cell lines expressed high levels of the molecules, MHC class I, MHC class II and CD45 (Hooshmand-Rad et al. 1993). However, in this study, only a few cell lines were used and limited monoclonal antibodies against cell surface molecules were available. Consequently, the results do not give a reliable indication of the phenotype of *T. lestoquardi*-infected cells.

Leemans et al. (1999b; 2001) further confirmed these observations and found that *T. lestoquardi* infected monocytes/macrophages in sheep, which is similar to the situation with *T. annulata* species in cattle (Sager et al. 1998). Furthermore, it was demonstrated that a small portion of *T. lestoquardi*-infected cells expressed surface IgM (sIgM) (Leemans et al. 2001), which is considered to be characteristic of B cells (Gupta et al. 1998). Expression of sIgM was in contrast to findings with *T. annulata* and *T. parva* species in which sIgM expression was found to be lost (Baldwin et al. 1988; Spooner et al. 1989). CD8, a T-cell marker, was also found expressed on the surface of a small population of *T. lestoquardi*-infected cells (Leemans et al. 2001). The observation of CD8 and sIgM expression suggests that *T. lestoquardi* species may infect both B and T cells. It was also observed that the overall phenotypic profiles of *T. lestoquardi*-infected cell lines that established after *in vitro* infection of peripheral blood mononuclear cells (PBMC) with sporozoites were very similar to those of the *T. lestoquardi* lines that were derived *ex vivo* (Leemans et al. 1999b; Leemans et al. 1999a).

1.3 Comparison of *T. lestoquardi* and *T. annulata*

1.3.1 Similarities between *T. lestoquardi* and *T. annulata*

Although *T. lestoquardi* species was initially considered to be similar to *T. parva* (Wilde 1967), results of more recent studies suggest that it is closer to *T. annulata* species. The two species share a similar morphology (Kirvar et al. 1998a) and display an overlapping geographic distribution (Hooshmand-Rad et al. 1973). They also share the same tick vector, *H. a. anatolicum* (Hooshmand-Rad et al. 1973). Furthermore, infected cell phenotypes are similar (Hooshmand-Rad et al. 1993) and the parasites are serologically cross-reactive and show the cross-protection (Leemans et al. 1999b). These observations imply a close evolutionary relationship between the two species.

Using molecular analysis, Schnittger et al. (2000) further showed that the small-subunit ribosome RNA (srRNA) gene sequence identity between *T. lestoquardi* and *T. annulata* was 99.7%, with 99.0% being observed between *T. lestoquardi* and *T. parva*, which makes *T. lestoquardi* and *T. annulata* the most closely related species of *Theileria* parasites (Schnittger et al. 2000b).

1.3.2 Diagnosis of *T. lestoquardi* infection

The close relationship of *T. lestoquardi* and *T. annulata* makes it difficult to differentiate them from each other. Traditionally, to detect, salivary glands of infected ticks have been collected and stained with methyl green pyronin (MGP) to distinguish infected acini (Walker et al. 1979). However, this method was found unreliable and failed to distinguish two species in most cases (Kirvar et al. 1998a). A PCR method using specific primers which amplify a 785bp fragment of *T. lestoquardi* 30 kDa merozoite surface protein was developed and it can be used as a marker to distinguish *T. lestoquardi* from *T. annulata* species. The PCR method proved to be 100 times more sensitive than blood smear examination (Kirvar et al. 1998b).

1.3.3 Differences between *T. lestoquardi* and *T. annulata* species

Although *T. lestoquardi* and *T. annulata* are closely related species, differences still exist. It has been observed that *T. annulata* sporozoites infect PBMC obtained from

sheep, goats and cattle and establish infections in each species *in vivo*. However, although *T. lestoquardi* sporozoites infect PBMC obtained from sheep and goats and infect sheep and goats *in vivo*, they failed to infect cattle *in vivo* or their PBMC *in vitro* (Brown et al. 1998).

Leemans et al. (1999a) extended these observations and reported that *T. lestoquardi* readily infected sheep and caused severe disease, but did not infect cattle. *T. annulata* infected both cattle and sheep, and although cattle were severely affected, sheep showed only mild clinical symptoms and failed to develop piroplasms. Cross-protection studies showed that, while *T. lestoquardi* infection protected sheep against subsequent *T. annulata* invasion, prior infection with *T. annulata* did not prevent infection with *T. lestoquardi* and establishment of schizonts and piroplasms (Leemans et al. 1999a).

Although sheep PBMC could be infected by *T. annulata* sporozoites and appeared to have same development rate as *T. lestoquardi*-infected sheep cell lines, it was found that *T. annulata* did not infect PBMC from sheep previously inoculated with *T. lestoquardi* sporozoites (Leemans et al. 1999b). The reason for this is unclear. The authors suggest that PBMC surface may express different molecules in naïve and *T. lestoquardi*-infected sheep. Phenotype differences have been described between PBMC from naïve and *T. annulata*-infected calves (Preston et al. 1992).

The studies above suggest that, although *T. lestoquardi* and *T. annulata* are very closely related, a significant difference exists between the two species in their capacity to infect sheep, goat and cattle *in vivo* and PBMC *in vitro*.

1.4 Life cycle

T. lestoquardi is an intracellular parasite and completes its life cycle in the mammalian host by successively infecting lymphoid cells and erythrocytes (Soulsby 1982). Current knowledge of the life cycles of *Theileria* parasites have been derived mainly from studies of *T. parva* and *T. annulata* species. The two species have similar life cycles

(Fig.1.1) (Mehlhorn et al. 1984) and, because of its close relationship with *T. annulata*, *T. lestoquardi* species is believed to have similar life cycles to *T. annulata* and *T. parva*.

Infection of vertebrate hosts with *Theileria* parasites is initiated by the feeding of infected ticks. Invasion is not a simple process but involves a series of complex interactions between the parasite and host cells. The invasive stage must come into contact with the host cell, recognize and bind to it via one or more specific ligand-receptor interactions, and then enter and establish itself within the host cell (Mehlhorn et al. 1984).

During its life-cycle in the mammalian host, the parasite undergoes sequential development in cells of the lymphoid system and later in erythrocytes. Sporozoites rapidly gain entry to host lymphocytes and access the cytosol, where they differentiate to schizonts. Differentiation is associated with transformation of the infected cell to a state of uncontrolled proliferation (Fawcett et al. 1982). Each infected cell undergoes clonal expansion, which results in rapid spread of the infection throughout the lymphoid system and the rest of the body and, within a few days, brings about clinical signs identical to those associated with leucosis (Fawcett et al. 1982). In a proportion of infected cells, schizonts undergo further development into merozoites, which are released upon lysis of the infected cell. Merozoites invade erythrocytes where they differentiate to piroplasms, which are infective for ticks (Mehlhorn et al. 1984).

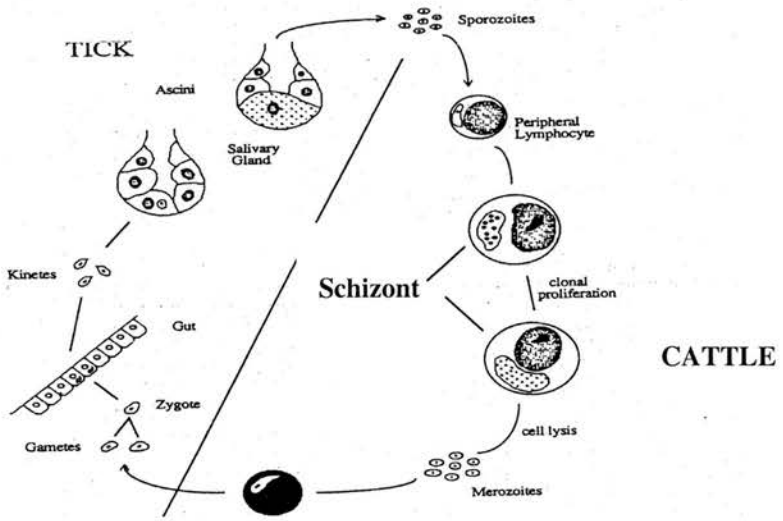


Fig.1.1 Life cycle of *T. parva* in cattle (Mehlhorn et al. 1984).

Piroplasms are ingested by feeding tick and differentiate to gametes. Gametogenesis and fertilization occur in the tick gut, leading to the development of the kinete within cells of the gut epithelium. The kinete gains access to the haemocoele and travels to the salivary gland. Sporogony characterized by massive multiplication occurs in selected cells within the salivary glands, giving rise to many thousands of sporozoites, which are injected during tick feeding (Shaw 1997). Transmission is transtadial, such that infection picked up by feeding larvae and nymphs is transmitted by nymphs and adults respectively (Mehlhorn et al. 1984).

As described above, the diseases caused by *Theileria* parasites are due mainly to the schizogonous stage in lymphoid cells. Schizont development in lymphocytes and characteristics of infected cells is described in the following section.

1.4.1 Schizont development in host cells

After inoculation by the tick, *T. parva* sporozoites enter lymphoid cells within 2-10 min. Following an initial binding event, the sporozoite is internalised through a zippering process (Shaw 1997) and, initially remains surrounded by the host membrane. The latter disintegrates within 24 hr of entry and is not retained as a parasitophorous vacuole (Schein et al. 1978) as in other *Apicomplexa,* such as *Plasmodium* (Pongponratn et al. 1991) and *Toxoplasma* (Mordue et al. 1999).

Arrays of microtubules appear around the sporozoite within 30 min of entry, which is the earliest detectable response of the host cell to the invasion, and microtubules become increasingly prominent over the next 2 hr (Fawcett et al. 1984). By 72 hours after infection, the parasite has enlarged considerably with the appearance of mitochondria and parasite cytoplasm has a higher concentration of ribosomes (Fawcett et al. 1982). Schizont nuclear division commences at this time. It consists of binary fission with appearance of a typical intra-nuclear spindle-apparatus at two poles of the nucleus. After a few cycles of nuclear division repetition, large schizonts of about 10-15 μm in diameter are formed and contain multiple nuclei about 1.5-2.0 μm in diameter (Fawcett et al. 1982).

Schizonts are morphologically similar in all *Theileria* species, with variation occurring only in the number of nuclei. *In vivo*, spherical schizonts form merozoites 8-10 days after infection with *T. annulata* or 12-14 days in the case of *T. parva* (Shaw 1997).

1.4.2 Morphological alterations in *Theileria*-infected cells

After *T. parva* sporozoite infection, host cells exhibit a series of morphological transformations: cell size increases approximately 3 times from normal size of 7 μm to 18-20 μm; nuclei alter with progressive loosening and dispersion of chromocentres and chromonemata, eventually converting to euchromatin; the host cell nucleolus undergoes a marked differentiation from its original ring-shape to a reticular configuration composed of several pale centres surrounded by materials of varying electron density and granularity; and the cell cytoplasm contain numerous clusters of ribosomes (Jura et al. 1983).

In addition, the host cell Golgi complex is also activated. The cisternae on its secretory face contain material of appreciable density and numerous irregular shaped vesicles with a content of similar density, accumulated around the periphery of the schizont. Although the vesicles are found close to the parasite membrane, there is no evidence of their fusion, or discharge of their contents into the parasite (Fawcett et al. 1982). On the basis of their morphology, the author interpreted the schizont associated vesicles as newly formed lysosomes from the host cell but no further study has been carried out since.

1.4.3 Association of *Theileria* schizonts with host cells and their division

A close association is observed between the microtubules of the centriole of the host cell and *Theileria* schizonts during cellular interphase. This association continues during the life of the schizont and results in the orientation of the schizont with the mitotic spindle formed from the microtubules (Hulliger et al. 1964). Examination of transformed cells in cytospin smears shows that the host chromosomes in most of the infected cells at prophase form a tight ring around the schizont. During the separation of the chromosome pairs and division of the cell, the association of the schizont with the spindle results in a passive and often uneven division of the schizonts (Fawcett et

al. 1984). As the extra-nuclear spindle elongates, the schizont becomes compressed between microtubular bundles, consequently, with dissolution of the host cell nuclear envelope, the parasite is incorporated into the mitotic spindle alongside the chromosomes. During anaphase, the daughter chromosomes are drawn to the poles as the chromosomal fibres shorten, but the schizont merely elongates further along with the microtubular bundles (Stagg et al. 1980).

With the separation of the host-cell chromosomes and cell division, schizonts are divided between the two daughter cells (Mehlhorn et al. 1984). Although the mechanism behind this is still unclear, it implies some kind of regulation between the parasite and host cell cycles, which guarantees a massive amplification of the parasite population.

1.4.4 Asynchronous DNA synthesis between host and parasite

Although *Theileria* schizonts induce host cells to proliferate, parasite and host cell cycles are asynchronous. It was found that, in the cultured *T. parva*-infected cells, the major component of parasite DNA synthesis occurs while the host cell is in early M-phase, with a minor component detectable during interphase. A distinct G2-phase appears to be missing from the parasite cell cycle because division of the schizonts immediately follows DNA synthesis. Parasite nuclear division appears to be synchronized with host cell metaphase (Irvin et al. 1982). The lack of synchrony of host and parasite S-phases suggests that the parasite regulates its DNA synthesis independently of the cell and this could have application in developing strategies for chemotherapeutic attack on the parasite.

1.5 *Theileria* transformation of leukocytes

1.5.1 Reversibility of *Theileria*-induced transformation

After *Theileria* infection, host cells are transformed and undergo uncontrolled proliferation. In several respects, the parasitized leukocytes display the characteristics of transformed cells, since they proliferate continuously in culture without the addition of growth factors or cytokines (Dobbelaere et al. 1988). Transformation of *Theileria*-

infected cell depends absolutely on the presence of the parasite and even after many years in culture is entirely reversible (Pinder et al. 1981). Following administration of the drug, BW720 (parvaquone) which induces parasite death, infected cells revert to a resting phenotype (Pinder et al. 1981), but undergo apoptosis in the absence of exogenous IL2 (Dobbelaere et al. 1988). It was also found that *Theileria*-transformed cells resemble cancer cells and produce tumours when injected into immunocompromised mice, sometimes showing more aggressive growth and metastatic potential than bovine lymphosarcoma cells (Fell et al. 1990; Irvin et al. 1975).

This host-parasite system is the only instance of eukaryotic organism that infects and transforms eukaryotic cells. The reversible nature of the transformation offers unique opportunities for the study of lympho-proliferation and cell cycle control.

1.5.2 Effect of transformation on cell signalling pathways

Upon invasion of a host cell, intracellular parasites escape destruction by the host immune system. However, another level of defence could be induced before host immune responses - the host cell can rapidly induce its own death, a process known as apoptosis (Strasser et al. 2000). Apoptosis is a regulated biochemical process that is normally induced when a cell is stressed or when it is in a state of uncontrolled proliferation (Strasser et al. 2000).

Theileria infection of the host cell has been found associated with modulation of leukocyte gene expression, including a number of genes encoding transcription factors that are implicated in the control of cell division or apoptosis. Some of these signal pathways will be described in detail in the following section.

1.5.2.1 MAPK and the activation of transcription factors

MAPK, the mitogen-activated protein kinases or extracellular signal regulated protein kinase (ERK), are kinases that transduce signals to the nucleus of the cell. This is achieved by regulating the activity of a range of transcription factors and other substrates. Members of the MAPK family have been found acting in pathways that control cellular differentiation and apoptosis (Robinson 1997). By stimulating

transcriptional activators, they induce alterations in gene expression, allowing appropriate responses to environmental stimuli. Two types of MAPKs, JNKs (stress-activated protein kinases) and members of the P38 family, have been identified that mediate responses to cellular stress (Robinson 1997).

JNKs have been shown to be constitutively activated in a parasite-dependent manner in *T. parva*-transformed T cells (Galley et al. 1997). Parasite-dependent JNK activation appears to result in activation of the transcription factors, AP-1 (homo- or heterodimers of c-jun) and ATF-2. In *T. annulata*-infected cells, AP-1 components are constitutively expressed and phosphorylated (Baylis et al. 1995). AP-1 activation is also suggested to be induced by metalloprotease-9 (MMP-9), a gene frequently found in metastasizing cancer cells (Baylis et al. 1995). Immunoblot analysis with antibodies specific for phosphorylated AP-1 confirmed that activated AP-1 translocated to the nucleus of parasitized, but not of cured cells. It was also found that phosphorylation of ATF-2 was parasite-dependent and was downregulated within 24 hrs of parasite elimination (Botteron et al. 1998).

Baumgartner et al. (2003) found that infection of bovine T cells and B cells with *T. parva* induces a transformed phenotype with characteristics comparable to leukemic cells. Both lymphocyte proliferation and activation of the transcription factor AP-1 are mediated by Src family protein tyrosine kinases (PTKs) in a parasite-dependent fashion (Baumgartner et al. 2003).

1.5.2.2 Src-related kinases

The protein tyrosine kinase Src is a kinase family that, in vertebrates, comprises eight members: Src, Fyn, Yes, Fgr, Hck, Lyn, Lck and Blk. The Src family associates with various intracellular membranes, where they catalyze the transfer of phosphate from ATP to a tyrosine residue within proteins (Neet et al. 1996). Src family also mediate early signalling events of many important T cell membrane receptors, including the antigen-specific TCR/CD3 complex and the CD4 and CD8 co-receptors (Chan et al. 1996). Their function is to convey extracellular signals through membrane-proximal compartments to cellular effector pathways, which induce cell DNA synthesis leading

to cell proliferation (Thomas 1997). Many Src-family members are considered to be proto-oncogenes and to be the targets of different viruses, which manipulate the signalling pathways of their target cells by modification of Src-family tyrosine kinase activity (Collette et al. 1997).

It has been suggested, on the basis of altered migrating patterns in SDS-PAGE that, in *T. parva*-transformed cells, Src-family kinases, Lck and Fyn, are constitutively activated (Eichhorn et al. 1994). Fich and others (1998) found that the proliferation of *T. parva* -transformed cells was blocked by tyrosine kinase inhibitors such as herbimycin A and genistein, which implies that *T. parva* - transformed cells rely on tyrosine phosphorylation for their continuous proliferation. Furthermore, it was observed that weak phosphorylation was found associated with Fyn and Lyn, whereas Lck was completely devoid (Fich et al. 1998). This suggests that Src-family kinases, Fyn and Lyn play critical roles, whereas Lck enzyme may not be important in transformation mechanisms in *T. parva*-infected cells.

While permanent activation of lymphocytes by *T. annulata* induces production and secretion of cytokines, cell to cell contact is required for growth of infected cells, which raises the possibility that surface receptor-activated Src kinases might be involved in this process. The proliferation of *T. annulata*-transformed B cells is associated with constitutive activation of the protein tyrosine kinase, Hck, which is apparently caused by exclusion of its negative regulator, Csk (Baumgartner et al. 2003).

1.5.2.3 NF-κB

Nuclear factor kappa B (NF-κB) is a transcription factor that plays an important role in inducing expression of cytokines and adhesion molecules, and genes that regulate proliferation and prevent apoptosis. NF-κB performs an integral role in the regulation of immune response genes (Pahl 1999) and it is essential for the innate immune response to pathogens (Elewaut et al. 1999).

In their constitutive form, NF-κB dimers are sequestered in the cell cytoplasm by the inhibitor protein, IκB, which suppresses the nuclear localization signal of NF-κB. In

response to appropriate stimuli, IκB undergoes a process of phosphorylation and degradation, leading to the release and activation of NF-κB. NF-κB then translocates to the nucleus, where it binds directly to specific decameric sequences (called κB motifs) that are located in the promotor and enhancer regions of a select number of genes. NF-κB proteins activate gene transcription by binding to sites in the target promoters of a range of genes including those encoding cytokines, growth factors, and immunoreceptors (Ghosh et al. 1998). NF-κB also induces expression of its own inhibitor, IκBα, and this results in a negative feedback loop, in which newly synthesized IκBα rapidly binds and inactivates NF-κB (Ghosh et al. 1998).

The NF-κB pathway has been studied in considerable details in *Theileria*-transformed cells and is one of the fundamental pathways contributing to parasite-induced transformation. In *T. parva*-transformed cells, NF-κB was found to be constitutively activated and this was dependent on the continuous presence of the parasite in the host cell cytoplasm, because elimination of the organism resulted in the disappearance of NF-κB from the host cell nucleus of *T. parva*-infected host-cells (Ivanov et al. 1989). Activation of NF-κB results in the expression of a number of κB-dependent genes, including several anti-apoptotic genes, such as c-*FLIP*, c-*iap*, and *xiap*. IκBs, the cytoplasmic inhibitors of NF-κB, are constitutively degraded in parasitized cells and NF-κB DNA binding complexes are found in their nuclei (Palmer et al. 1997).

The question of how NF-κB is activated in infected cells is still not clear. It has been found that stimuli that trigger NF-κB activation all converge onto a multi-subunit kinase complex called IKK (IκB kinase) and a modulating subunit called NEMO (IKKγ). Interference with IKK and NEMO blocks parasite-induced NF-κB activity and results in rapid apoptosis (Heussler et al. 1999). It has also been suggested that *Theileria* might short-circuit the NF-κB activation pathway, as several drugs that interfere with upstream components of the NF-κB activation cascade in other systems fail to block NF-κB activity. Host cell IKK and NEMO have been found to cluster on the *Theileria* parasite surface, which demonstrates that the parasite can accommodate the different host cell IKK and NEMO components (Heussler et al. 2002).

To understand transformation mechanism employed by *Theileria* parasites further, it will be important to identify the schizont surface proteins that induce IKK aggregation and activation. However, a direct association between parasite proteins and host IKK and NEMO components has not been found.

1.5.2.4 PI3-K/PKB

PI3-K/PKB is another signalling pathway that is involved in regulation of proliferation and survival in many cell types. On stimulation with growth factors, PI3-K is activated and phosphorylates inositol lipids in the plasma membrane that attract the kinase PKB (also named Akt). On association with these lipids, PKB is activated by PDK1-mediated phosphorylation and targets several proteins involved in apoptotic pathways, including Bad, caspase-9, and transcription factors and IKK (Cardone et al. 1998; Klippel et al. 1998).

Both PI3-K and PKB were found to be activated in a *Theileria* parasite-dependent manner. Interference with the PI3-K/PKB pathway using a specific PI3-K inhibitor arrested growth, but did not induce immediate cell death of parasitized leukocytes (Baumgartner et al. 2000; Heussler et al. 2001). Although a firm link between surface receptors and PI3-K activation has not yet been established in *Theileria*-transformed cells, there are indications that PI3-K activity occupies an essential function in *Theileria*-induced transformation, and PI3-K inhibitors, wortmannin and LY294002, blocked proliferation of *Theileria*-transformed cells (Heussler et al. 2001).

In summary, it has been proposed that different signalling pathways and many different families of kinases are involved in host cell transformation after *Theileria* parasite infection, some of which have been identified. It is believed that mechanisms of transformation are complicated; although it is realized which host cell signalling pathways contribute to *Theileria*-induced transformation, it remains unclear how the parasite interferes with these signalling pathways.

The challenge in understanding how *Theileria* parasite interferes with signalling pathways is to identify parasite molecules that are directly involved in the pathways. A number of parasite molecules have been identified with potential roles in the interaction with the host cell and these are discussed in the next section.

1.5.3 *Theileria* molecules potentially involved in parasite-host cell interactions

1.5.3.1 Casein kinase II (CK2)

CK2, required for cell viability and cell cycle progression, is highly pleiotropic and phosphorylates over 160 proteins at sites specified by multiple acidic residues. It is especially elevated in proliferating tissues, which are either normal or transformed (Pinna 1997). CK2 was believed to contribute significantly to protein phosphorylation, which plays an important role in the regulation of cellular growth and in the genesis of tumours (Pinna et al. 1997).

CK2 increases significantly at transcriptional, translational, and functional levels in the schizont stage of *T. parva*-infected lymphocytes, and it has been suggested that this kinase might be an important element in the signal-transducing pathways activated by *T. parva* (ole-MoiYoi et al. 1989). A *T. parva* CK2 catalytic α-subunit was cloned and it was proposed that the protein could be inserted into the parasite plasma membrane or be secreted into the host cell cytoplasm (ole-MoiYoi 1995). Using transgenic mice in which the murine CK2-α subunit of the enzyme was put under the control of a globulin promoter, it was shown that CK2-α could function as an oncogene (Seldin et al. 1995).

A CK2-α subunit gene has also been cloned from *T. annulata* (Biermann et al. 2003). The gene is single copy, contains no introns, and its transcription is restricted to the schizont stage. With indirect immunofluorescence using anti-parasite CK2-α serum, it was found that the protein is confined to the schizonts within the host cell (Biermann et al. 2003). This suggests that the CK2-α subunit is possibly not secreted by the schizont. If so, its direct involvement in the signalling pathway seems unlikely.

1.5.3.2 TashAT

There is evidence that, when *T. annulata* parasites are induced to differentiate to the extracellular merozoite stage, the rate of host cell proliferation is significantly reduced (Shiels et al. 1992). It has been proposed that elucidation of how proliferation is negatively controlled during differentiation could lead to identification of factors that control host cell division (Shiels et al. 1992).

Investigation of negative gene regulation during differentiation from the schizont to the merozoite in *T. annulata* identified a gene designated TashAT2. The predicted amino acid sequence of TashAT2 bears AT hook DNA-binding motifs and may be transported from the parasite to the host nucleus (Swan et al. 1999). Two additional genes, TashAT1 and TashAT3, which are closely related to TashAT2, have recently been identified (Swan et al. 2001b). All three genes encode AT hook DNA-binding motifs and are very closely related in sequence. TashAT2 has three AT hook motifs, whereas TashAT1 and TashAT3 have four, implying that the latter two have a stronger affinity for DNA than TashAT2 (Swan et al. 2001a).

These three genes of the TashAT family possess putative signal sequences as well as nuclear localisation signals, demonstrating that they contain the structural information that would allow them to be secreted from the schizont and transported into the host cell nucleus. Immunoblot analysis of host and parasite nuclear fractions with specific antiserum reveals two bands of 180 KDa and 66 KDa (Swan et al. 2001b). Immunofluorescence studies indicate that anti- TashAT1/3 serum has strong reactivity within the host cell nucleus. It was also found that TashAT genes express their products differentially during the development from schizonts to merozoites (Swan et al. 2001b).

1.5.3.3 TashHN

TashHN gene is an integral part of TashAT cluster found in the *T. annulata* genome and is also involved in regulation of host gene expression during schizont differentiation to the merozoite. TashHN displays three forms, the largest of which is

enriched in the host nuclear fraction and appears to be phosphorylated. TashHN encodes a predicted polypeptide consisting of 332 amino acid residues and a predicted molecular weight of 33.8 kDa. The predicted polypeptide contains a putative N-terminal signal sequence cleavable at position 23 and several nuclear localisation signals, which suggests that TashHN may target the host nucleus. In addition, there is a putative cyclin A docking site at position 124, two potential protein kinase C sites and multiple CK2 phosphorylation sites, which suggests TashHN may be a substrate for mammalian cell cycle associated kinases (Swan et al. 2003).

Immunofluorescence analysis using a rabbit antiserum raised against a TashHN fusion protein revealed that TashHN locates to the parasite and to the host cell nucleus. It was found that TashHN expression increased in an attenuated *T. annulata* cell line and decreased during differentiation from schizont to merozoite, which is similar to TashAT1, 2 and3 proteins (Swan et al. 2003). The importance and functional role of TashHN during differention of schizonts to merozoites remains to be determined.

1.5.3.4 SuAT1

The *T. annulata* SuAT1 gene was identified from a schizont genomic λgt11 expression library by screening with a concatenated double-stranded CAT1 DNA probe (Shiels et al. 2004). The SuAT1 gene encodes 558 amino acids and contains an AT hook motif with an RGRP core that is identical to AT hook domains previously identified for the TashAT proteins (Swan et al. 2001b). The SuAT1 gene also encodes three potential nuclear localization signals. Using antiserum raised against the SuAT1 gene, SuAT1 was found to be localized to the nuclei of schizont-infected cells, which indicates that SuAT is secreted and transported to host-cell nuclei. However, it was found the mRNA and protein expression profiles of SuAT are distinct from those of TashAT genes (Shiels et al. 2004). During the time of differentiation from schizont to merozoite and in the merozoite stage, SuAT1 in the leukocyte nucleus was detected at elevated levels, whereas TashAT genes were down-regulated (Swan et al. 2003). This suggests that SuAT1 is unlikely to be

involved in the stimulation of the host cell proliferation but may be associated with the inhibition of host cell division. Transfection of an uninfected cell line demonstrated that SuAT1 can modulate cellular morphology and change the expression pattern of a cytoskeletal polypeptide (Shiels et al. 2004).

1.5.3.5 *Theileria* cyclophilin

Cyclophilins are a class of peptidyl-prolyl cis-trans isomerases, which are ubiquitous and highly conserved in eukaryotes (Gothel et al. 1999). Cyclophilin proteins within the same cell can have different subcellular localizations and distinct functions. A number of cyclophilins entering the secretory pathway have been described, most of which are either soluble proteins located in organelles of the secretory pathway or are secreted (Gothel et al. 1999). There is evidence of a role for cyclophilins secreted by protozoan parasites in host cell invasion (Moro et al. 1995). One location for the cyclophilins is the endoplasmic reticulum (ER), an organelle that is a fundamental component of the eukaryotic secretory pathway (Wiser et al. 1999).

In screening a *T. annulata* cDNA library, a clone (Tacyp) with significant homology to cyclophilin genes was found (Swan et al. 1996). The N-terminus of Tacyp contains a stretch of hydrophobic residues typical of signal sequences, implying that the protein is either secreted or located on the parasite surface. Northern blot analysis showed that Tacyp is constitutively expressed during schizont and merozoite stages, and that its expression is elevated in the piroplasm stage (Swan et al. 1996).

A cyclophilin gene homologue (Tpcyp) has also been found in a *T. parva* piroplasm cDNA library (Ebel et al. 2004). Tpcyp gene encodes a 227 amino acid residue product and its C-terminal 165 residues are closely related to a wide variety of cyclophilins and constitute a complete cyclophilin domain. The region has 70% amino acid identity with a plant cyclophilin homologue of *Arabidopsis thaliana*, but only 49% identity with Tacyp of *T. annulata* species (Ebel et al. 2004). This suggests that Tpcyp and Tacyp belong to different cyclophilin families. Northern blot analysis revealed that both *T. parva* schizont and piroplasm stages transcribe the Tpcyp gene, with the schizont stage showing high levels of transcription. Cell-free *in vitro*

transcription/translation provided evidence that Tpcyp is membrane-anchored via its non-cleaved signal sequence and that the C-terminal portion is translocated across the membrane (Ebel et al. 2004). However, whether Tpcyp is located in the ER or transported to other cellular compartments has not been determined.

From these observations, it is clear the parasite secretes antigens to the cytoplasm and expresses antigens on its surface. It is believed that some of these antigens interfere with one or more components of the signalling pathways that govern the proliferation of host cells. Genes encoding several of these molecules, including CK2 and Tpcyp in *T. parva* and the TashAT family and TashHN gene in *Theileria* parasites, have been cloned and their products have been characterized. However, the exact process by which *Theileria* secreted proteins interfere with signalling pathways remains to be determined and, importantly, more proteins involved in these pathways need to be identified.

Having infected host cells, the parasite ensures completion of its life cycle in the bovine host through induction of host cell proliferation as described above. However, in response to proliferation of *Theileria*-transformed cells, the host deploys its immune system to eliminate the pathogen. The major protective mechanism in *Theileria*-infection has been found to be the cellular response and the effector cells are mainly $CD8^+$ T cells (Pearson et al. 1979; McKeever et al. 1994).

1.6 Cytotoxic T lymphocyte (CTL) responses to *Theileria* parasites

Several features of immunity to these parasites indicate that it is against the schizont stage. Animals can be immunised by inoculation with schizont-infected cell lines and development of this stage is necessary for induction of immunity (Morrison et al. 1996). In addition, immune animals usually develop a schizont parasitosis before clearing challenge infections (Eugui et al. 1981). Because the schizont is inaccessible to antibody, humoral immune mechanisms do not play a significant role in controlling schizont parasitosis. Indeed, transfer of serum from immune animals, whilst containing

anti-schizont antibodies, does not passively confer immunity to a naïve recipient (Muhammed et al. 1975).

Cellular immune mechanisms against the *T. parva* are now well characterized. PBMCs of immune cattle proliferate in the presence of autologous *T. parva*-infected cells and a proportion of responding cells exhibit parasite-specific killing activity. Specific cytolytic activity can also be observed directly in PBMCs derived from immune cattle under challenge with the parasite, around the time that the infection is eliminated (Emery et al. 1982). In *T. parva* infected cattle, the cells responsible for the cytolytic activity are mainly $CD8^+$ T cells (Goddeeris et al. 1986). Immune responses have been examined directly in lymph nodes undergoing challenge with *T. parva*. By cannulating the lymphatic vessels and collecting the emerging lymphoid cell populations, it was determined that killing activity is present in the immune lymph node from day 7 after challenge. At the height of the response, as many as 5×10^{10} $CD8^+$ T cells can be isolated from an overnight collection of lymph, of which one in 30 may be a cytotoxic lymphocyte (McKeever et al. 1994). The large numbers of CTL made it possible to conduct transfer experiments in twin calves in order to evaluate the role of CTL in protection. The enriched $CD8^+$ fractions collected after challenge of one calf were inoculated daily into the naïve twin over the period of the response. It was found that, challenge infections were cleared without treatment and parasites were not detected in either of these animals at any stage after challenge (McKeever et al. 1994) which provide the evidence that $CD8^+$ T cells are responsible for the cytolytic activity in *Theileria* infection.

The lysis of *T. parva* infected cells by immune PBMCs is dependent on major histocompatibility complex (MHC) specificity being shared between effector and target cells. The MHC-restricted CTLs are highly specific, as they recognize only *T. parva* schizont-infected cells and are often specific to a particular parasite strain (Morrison et al. 1995a).

1.6.1 Influence of host MHC and parasite strain

Lysis of *T. parva* infected cells by immune PBMCs is restricted by MHC class I. This MHC class I restriction was confirmed by blocking of target cell lysis with monoclonal antibody w6/32, which reacts specifically with a monomorphic determinant on bovine class I MHC molecules (Goddeeris et al. 1986).

Strain-specificity of *T. parva* specific CTL responses has been best studied with the Muguga and Marikebuni stocks (Minami et al. 1983). Muguga has undergone an indeterminate number of laboratory passages since its isolation and is relatively homogeneous with respect to a number of antigenic markers. Marikebuni has been passaged only twice since its isolation and is known to contain at least five antigenically distinct components (Goddeeris et al. 1990). To investigate protection against infection of different stocks, it has been shown that, cattle immunized with Marikebuni stock are invariably protected against challenge with both stocks, as well as a variety of other stocks, whereas only a proportion of animals immunized with Muguga mount a protective response against the Marikebuni stock (Irvin et al. 1983).

To determine whether MHC phenotype of cattle could affect the parasite strain specificity of immunity to *T. parva* infection, Goddeeris et al. (1990) investigated the parasite strain specificity of *Theileria*-specific CTL clones derived from cattle of different class I MHC phenotypes. It was found that CTL clones restricted by the same MHC class I determinant had similar parasite strain specificities, whereas clones restricted by different MHC class determinants exhibited different parasite strain specificities, even when they originated from the same animal (Goddeeris et al. 1990). These observations suggest that differences in the strain specificities of CTL are determined by the MHC class I phenotype of the immunized animal. To define the basis of the parasite strain specificity of CTL responses to *T. parva*, it was revealed that, in most animals, the CTL response was restricted entirely by the products of one MHC haplotype and, where tested, to one MHC molecule (Taracha et al. 1995). However, none of the parasite antigens responsible for the $CD8^+$ T cell response have been described and the mechanisms of antigen processing and presentation by *Theileria*-infected cells are unclear. However, relevant information can be obtained from other studies on antigen processing and presentation.

1.7 General mechanisms of antigen processing and presentation

1.7.1 The T cell and MHC

Antigen-specific immune responses comprise two distinct types: humoral and cellular immune responses. The effector components of the humoral response are antibodies secreted by B lymphocytes and those of the cellular responses are T lymphocytes. In general, antibodies recognize native antigen molecules in solution, whereas antigen-specific T cells recognize antigen in conjunction with molecules encoded by the MHC genes (Schwartz 1985).

The two main classes of MHC molecules, class I and class II, have been characterised extensively, are morphologically distinct and present peptides from different sources. Processing of antigens for class I and class II presentation follows different pathways within antigen-presenting cells (APC). MHC class I presents endogenous peptides for recognition by T cell receptors (TCR) of $CD8^+$ T cells while class II presents peptides of exogenous origin to TCR of $CD4^+$ T cells (Townsend et al. 1989).

1.7.2 Antigen processing and presentation

1.7.2.1 Generation of peptide epitopes

Degradation of antigens is an essential step in the generation of MHC class I-presented epitopes. It is supposed that protein degradation is highly specific and tightly regulated to prevent non-specific destruction of essential self-proteins. It has been shown that proteasomes mediate the majority of endogenous cytoplasmic protein degradation (Eggers et al. 1995). Proteasomes, referred to as multicatalytic proteases, contain a barrel-shaped 20S core particle that is proteolytically active and can degrade proteins in an ATP-independent fashion (Eggers et al. 1995).

Antigens are passed through the proteasome within the cell, which breaks the antigen up into manageable peptides between 13 and 18 residues long (Goldberg et al. 1992).

Degradation of endogenous proteins is modulated in various ways. Some proteins are rapidly degraded because they contain PGST sequences, which are rich in proline, glutamine, serine, and threonine (Rogers et al. 1986).

Studies of the P60 protein from *Listeria monocytogenes* demonstrate that there is a direct correlation between antigen degradation rates and the generation of T cell epitopes (Sijts et al. 1997). Other proteases within the endoplasmic reticulum (ER) and the cytoplasm may also be involved in degradation (Glas et al. 1998).

1.7.2.2 Transport of peptides

Transportation of processed peptides from the cytoplasm to the ER is mainly completed by the transporter associated with antigen processing complex (TAP). It is still not clear how peptides that are released from proteasomes reach the TAP peptide transporter in the ER membrane. Peptides derived from cytoploasmic proteins are either undetectable or are present in very low amounts in the ER. It has been proposed that processed peptides are protected from further degradation by chaperone proteins, such as heat shock proteins (hsp70 and hsp90) (Srivastava et al. 1994).

The TAP complex consists of two main sub-units, TAP 1 and 2, both of which are MHC-encoded. Each TAP subunit has a hydrophobic N-terminal region with multiple predicted transmembrane domains, and a cytosolic C-terminal ATP-binding domain (Watts et al. 1999). Peptide binding by TAP is ATP-independent, while translocation is ATP-dependent (Neefjes et al. 1993).

1.7.2.3 Peptide processing

Processed peptides must pass into the lumen of the ER before they can bind to MHC I molecules, which are assembled in this organelle. Proper assembly of the MHC class I-peptide complex is required for stable surface expression of class I molecules.

Mature MHC I complexes consist of three noncovalently associated components: a 45-kDa heavy chain (HC), β_2- microglobulin (β_2m), and a short processed peptide. Two

domains of HC (α_1 and α_2) fold together to form a groove that binds and displays peptides (Hosken et al. 1990). Assembly occurs in the ER, where newly synthesized MHC class I heavy chains and β_2m form dimers. Without processed peptide, the association of MHC I molecules leads to an unstable complex and may either be inefficiently transported to the cell surface or degraded in the ER compartment (Hosken et al. 1990). Using peptide aldehyde inhibitors, it was demonstrated that proteasomes degrade short-lived and long-lived proteins, and that, in the absence of proteasome-mediated protein degradation, MHC I molecules remain in the ER, starved of peptides (Rock et al. 1994).

A major chaperone involved in MHC I assembly is calnexin. This is an ER-retained transmembrane protein that shares the property of binding to monoglucosylated N-linked glycans with a homologous soluble ER protein, calreticulin (Hammond et al. 1995). After the heavy chain and β_2m of MHC I are co-translationally transported into the ER, the heavy chain is unstable and prone to degradation so the chaperone protein calnexin is required to stabilise this. As soon as β_2m binds calnexin, calnexin is replaced with calreticulin. An additional molecule, tapasin, links the MHC/calreticulin molecule to the TAP complex and is thought to help loading of peptides onto the binding site (Engelhard 1994).

After assembly, MHC I molecules transit through the Golgi apparatus, where N-linked carbohydrates are modified. They are then rapidly transported by the default exocytic pathway to the plasma membrane where they display their bound peptides to $CD8^+$ cells (Engelhard 1994).

1.8 *Theileria* schizont molecules as potential vaccine candidates

Hsps are widely distributed in nature and are among the most highly conserved molecules of the biosphere. Hsps perform important functions as chaperones in the folding and unfolding or translocation of proteins (Kaufmann 1996). They are also involved in antigen presentation, interactions of intracellular pathogen and host cell and also act as immunogenic antigen in CTL immune response (Himeno et al. 1996;

Kaufmann 1996). In murine infections with *Helicobacter pylori*, immunization with hsp60 induced significant protection (Ferrero et al. 1995). In addition, adoptive transfer of Yersinia-hsp60-specific T cells induced protection against infection with *Yersinia enterocolitica* (Noll et al. 1994), while immunisation with hsp60 or hsp70 of *Histoplasma capsulatum* induced protection against infection with *H. capsulatum* (Gomez et al. 1995).

In *Theileria* parasites, hsps, including hsp70, mhsp70 and hsp90, have been cloned and some of their functions have been characterised (Gerhards et al. 1994; Mason et al. 1989; Schnittger et al. 2000a).

1.8.1 Tamhsp70

A *T. annulata* mitochondrial hsp70 (Tamthsp70) has been cloned from cDNA library and found to be most closely related to a previously reported mitochondrial hsp70 gene of *Eimeria tenella*. Tamthsp70 has an ORF coding for a protein of 681 amino acids with a predicted molecular weight of 74.3 kDa. The Tamthsp70 mRNA is expressed within the sporozoite, schizont, and merozoite stages of the parasite, which suggests that it is constitutively transcribed throughout the life cycle. The gene exhibits a mitochondrial targeting sequence and several sequence motifs common to mitochondrial hsp70 and prokaryotic dnaK proteins (Schnittger et al. 2000a).

Localization of Tamthsp70 in schizont-infected cells was examined by indirect immunofluorescence assay (IFA) and immunoelectron microscopy (IEM). It is found that Tamthsp70 present predominantly in schizont mitochondria, distributed mainly in close proximity to the inner mitochondrial membrane, and also in host cell cytoplasm. The presence in host-cell cytoplasm suggests that it is transported across the parasite membrane into the host-cell cytoplasm. Searching the Tamhsp70 amino acid sequence, it was found that it has peptide motifs with potentially very high binding affinity to bovine class I A20. This suggests that Tamhsp70 is a potential antigen target for MHC I presentation in host CTL immune responses (Schnittger et al. 2000a).

1.8.2 Hsp90

Hsp90 of *T. parva* has homology of more than 62% to hsp90 of other organisms at the protein level. The gene is expressed as a protein of 87 kDa in both the sporozoite and schizont stages of *T. parva*. In the schizont stage, the protein is upregulated upon heat shock. No evidence of hsp90 secretion has been found. When examined by immunofluorescence microscopy, it appears to be distributed uniformly throughout the schizont cytoplasm without being obviously asociated with the schizont membranes. This localisation pattern is in agreement with the cell-free expression analysis where the protein is not translocated across membranes derived from ER (Gerhards et al. 1994).

1.8.3 Tahsp70

Hsp70 gene of *T. annulata* (Tahsp70) has been cloned from DNA library. The gene is expressed constitutively in sporozoites, piroplasms and in schizonts. In schizonts, heat shock induces an increase in the expression level of Tahsp70 mRNA. The gene sequence of 2551 bp contains a single open reading frame coding for a protein of 646 amino acids with a calculated molecular weight of 71 kDa. Comparison of Tahsp70 protein sequence with that of *Drosophila melanogaster* hsp70 homologue shows 65% identity. The homology is less evident in the most C-terminal portion of the protein although the last 9 amino acids are identical (Mason et al. 1989). Immunoelectron microscopy using a rabbit antiserum raised against Tahsp70 407 C-terminal amino acid residues showed that Tahsp70 is distributed uniformly in the schizont cytosol and is not obviously present in the host cytoplasm, which suggests Tahsp70 is not secreted by schizonts (Mason et al. 1989).

As described above, in response to *Theileria* parasite infection, the animal initiates immune responses to eliminate infected parasites. There is direct evidence that, in *T. parva*-infected animals, $CD8^+$ CTL plays a major role in killing the parasite living within the host cell. Given the pathway for class I processing, it is likely that secreted or surface antigens are targeted for recognition by immune T cells. These observations highlight the potential for employing secreted antigens recognized by $CD8^+$ CTL for

immunization. A few *Theileria* hsps with potential vaccine candidate have been identified, but their functions have not been determined. It is still not known which peptides or antigens are involved in the CTL responses. No information is available on CTL responses against *T. lestoquardi* and their peptide specificity.

The genomes of *T. annulata* (http://www.sanger.ac.uk/Projects/T_annulata/) and *T. parva* (http://tigrblast.tigr.org/ufmg/index.cgi?database=t_parva) have now been sequenced and that of *T. annulata* has been annotated. The completed *Theileria* genome sequences will assist identifying genes potentially involved in host immune responses and signalling pathways. The purpose of this study was to identify secreted proteins of *T. lestoquardi* schizonts, with a view to understanding their role in immunity and transformation.

Project strategy

T. lestoquardi parasites invade and transform lymphoid cells in vivo. Each infected cell undergoes clonal expansion, which results in the rapid spread of infection throughout the lymphoid system and causes animal death. The disease is mainly due to schizont stage in *Theileria* infection. We hypothesize that schizont secreted proteins are involved in transformation of the infected cell and are also potential antigens presented by the infected cell to $CD8^+$ T cells. Therefore, schizont secreted proteins are important both for infected cell transformation and host immune responses. However, since little is known about secreted proteins of *Theileria* in general, the purpose of this study was to identify proteins secreted by schizonts of *T. lestoquardi*.

A proteomic strategy was adopted, based on two-dimensional electrophoresis (2DE) and mass spectrometry. The strategy involved axenic culture of schizonts purified from metabolically labelled infected cells and identification of labelled proteins in culture supernatants by 2DE. Corresponding spots were located in 2DE gels of whole schizonts and excised for analysis by MALDI-TOF, Q-TOF and N-terminal sequencing. The following chapters outline development of the methods necessary for this strategy, the outcome of its application and interpretation of its results.

Chapter 2: An efficient method for *T. lestoquardi* schizont purification

2.1 Introduction

Elucidating the mechanisms of *T. lestoquardi* schizont-induced transformation and the specificity of the host immune response awaits the identification of putative oncogenes and immune target antigens. Difficulties associated with obtaining pure schizont material have limited progress in this regard. The strategy adopted by this study required isolation of viable schizonts from metabolically labelled cells to a high degree of purity.

A number of methods for separation of intracellular protozoan parasites have been developed previously. Several gradient media, including Nycodenz (Carter et al. 2003) and Percoll (Knight et al. 1998) have been used and malaria parasites have been purified efficiently from infected erythrocytes using Nycodenz and Percoll gradients. For *Toxoplasma* parasites, a filtration method based on polycarbonate nucleopore membranes and Nycodenz gradient media has been successfully used to isolate tachyzoites from infected cells (Coppens et al. 2000).

Several reports have described treatments for the release of schizonts from *T. parva*-transformed cells. When parasitised cells are lysed by aerolysin extracted from *Aeromonas hydrophila*, schizonts released from host cells retain their normal morphology (Sugimoto et al. 1988). These authors also found that schizonts could be isolated with high purity and yield using Percoll gradient media (Sugimoto et al. 1988). An improved method for purification of *T. parva* schizonts was developed by Goddeeris et al. (1991), in which Nyzodenz gradient media was used. Numerous intact schizonts were obtained, with only occasional unlysed host cells and host-cell nuclei. The latter method was considered simpler and more efficient than the one described by Sugimoto et al. (1988).

Ultra structural studies have provided evidence for an association of *T. parva* schizonts with host cell microtubules (Fawcett et al. 1984; Shaw et al. 1991), and this may prevent efficient separation of schizonts from host cellular components. In a further modification, Baumgartner et al. (1999) used a microtubule inhibitor, nocodazole, to disrupt the microtubule association with schizonts. Nocodazole belongs to the group of colchicine-site-binders that interact with tubulin dimers and inhibit their assembly into microtubules, resulting in the loss of most cytoplasmatic microtubules, including spindles (De Brabander et al. 1976). Using nocodazole allowed further improvement of the schizont purification providing *T. parva* schizonts with higher quantity and quality (Baumgartner et al. 1999).

However, these observations were made with *T. parva* rather than *T. lestoquardi* parasites and available literature on *T. lestoquardi* species is limited. Because of the different characteristics between schizonts and infected cells of *T. parva* and *T. lestoquardi* (Kirvar et al. 1998b; Schnittger et al. 2000a), it was considered that existing methods for *T. parva*-infected cell lysis and schizont purification might not be suitable for *T. lestoquardi* parasites. This chapter describes the establishment of an efficient method for lysis of *T. lestoquardi*-infected cells and release and purification of schizonts.

2.2 Materials and methods

2.2.1 Establishment of *Theileria lestoquardi*-infected leukocytes (TLL)

2.2.1.1 Preparation of sheep peripheral blood mononuclear cells (PBMC)

Sheep blood (15 ml) was collected in heparinised evacuated tubes (Vacutainer TM, Becton Dickinson Ltd, UK). The blood was centrifuged at 1300 × g (Sorvall® RT7 Plus, Jouan, UK) for 15 min at 4 °C (no braking). The buffy coat cells were collected and resuspended in 5 ml of 10U/ml heparin in complete Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies Company) supplemented with 5% foetal calf serum (FCS), 2 mM L-glutamine, gentamycin at 50 µg/ml. The cell suspension (4 ml) was overlaid on 6 ml of lymphoPrep (Axis-Shield POC AS) and centrifuged at 670 × g (Sorvall® RT7 Plus) for 30 min at 4 °C. The PBMC enriched interface was transferred to 10 ml Hanks' buffered saline solution (HBSS) supplemented with 10U/ml heparin and pelleted at 500 × g (Sorvall® RT7 Plus) for 10 min. The pellet was then resuspended, washed in 10 ml HBSS supplemented with 10U/ml heparin and finally resuspended in complete IMDM at 2 $\times 10^6$ cells/ml for infection with *Theileria* sporozoites.

2.2.1.2 Infection of sheep PBMC with *T. lestoquardi* sporozoites

Infection of sheep PBMC by *T. lestoquardi* sporozoites was carried out essentially according to the method described by (Brown 1983) and (Goddeeris et al. 1988). Ground up tick supernatant (GUTS, 4 te/ml, te- tick equivalent) containing *T. lestoquardi* sporozoites was kindly provided by Dr Alan Walker, Centre for Tropical Veterinary Medicine (CTVM) of Edinburgh University, and diluted gradually with doubling dilutions to 2, 1, 0.5, 0.25 te/ml in complete IMDM medium. One ml of GUTS dilutions at 0.5 and 0.25 te/ml were mixed with equal volumes of PBMC at 2 $\times 10^6$ cells/ml in 24-well plate wells (Costar, USA) and incubated for 1-2 weeks at 37 °C in a humidified atmosphere of 5% CO_2 in air.

T. lestoquardi-infected leukocytes (TLL) were detectable under an inverted microscope (Olympus) within 7-10 days as large blasting cells. To confirm

establishment of infection, cell suspensions (50 µl at 2 $\times 10^5$ cells/ml) were spun down at 500 rpm for 5 min in a Cytospin3 cyto-centrifuge (Shandon, UK) for preparation of cytospin smears. The smears were air dried, fixed in methanol for 5 min and stained with 5% Giemsa (Fluka, Switzerland) in staining buffer (7.6 mM Na_2HPO_4, 2.94 mM KH_2PO_4, pH 7.2) for 15 min. They were washed with distilled water (dH_2O) 5 min and observed under a light microscope (Olympus). Once numbers of infected cells reached 10^6 cells/ml, cells were harvested, subcultured and maintained in T75 (75 cm^2) flasks in complete IMDM medium in the incubator at 37 °C.

2.2.1.3 Establishment of cell growth curves

To establish the optimal time for harvesting after sub-culturing TLL, cell growth rates were assessed and growth curves derived. Using a T75 flask containing 10 ml complete IMDM medium, cells were cultured at a density of 2 $\times$ 10^5 cells/ml and counted every 24 hrs. At each interval, a 50 µl aliquot of cell suspension was added to 50 µl of 0.1% nigrosin (Sigma) in phosphate-buffered saline (PBS) and mixed thoroughly. A Neubauer counting chamber was charged with the cell/nigrosin mixture by capillary action and cells were counted in the centre square and 4 corner squares (0.1 mm^3 / square) and cell numbers calculated as follows:

Total cells = the average number per square $\times$ 10^4 $\times$ dilution factor $\times$ total cell volume

A curve was drawn with cell culture time (x) plotted against cell population (y) to show the cell growth rate.

2.2.2 Cell lysis with aerolysin

2.2.2.1 Preparation of *Aeromonas hydrophila* (strain AH-1) aerolysin

Aerolysin obtained from *A. hydrophila* is able to lyse eukaryotic cells through the formation of discrete transmembrane pores (Asao et al. 1984). A freeze-dried *A. hydrophila* AH-1 pellet (NCTC, UK) was resuspended in 0.5 ml Tryptose phosphate broth and inoculated on brain heart infusion agar plates. After overnight

culture at 30 °C, a typical colony was picked and inoculated in 10 ml DYR medium (Davis minimal broth + 1% Yeast extract + 1% RNA). After a further overnight incubation at 30 °C, the culture was added to 1litre (L) DYR medium and cultured for 9 hrs at 30 °C with shaking.

Aerolysin was purified by RNA precipitation essentially as described by Asao et al (Asao et al. 1984). The bacterial culture was centrifuged at 15, 000 × *g* for 10 min at 4 °C using a SW41 rotor in a Beckman J2-21ultracentrifuge. The supernatant was transferred to two 500 ml flasks, and 100 mg/ml RNA solution (pH6.0) in dH_2O was added to a final RNA concentration of 0.3 mg/ml. The pH of the solution was adjusted to 4.0 by adding 1 M HCl, and the solution was incubated at 4 °C for 2 days to allow the RNA- haemolysin complex to precipitate by acidification (Iwasaki et al. 1978). After precipitation for 2 days, the supernatant was discarded and the precipitated material was resuspended, transferred to 50 ml tubes and pelleted by centrifugation at 5, 000 × *g* (J2-21 centrifuge, JA20 rotor, Beckman Coulter) for 30 min at 4 °C. After centrifugation, the supernatant was removed and the aerolysin-containing precipitate was dissolved in 20 ml of 0.05M Tris-HCl (pH 7.5) containing 1 M urea.

2.2.2.2 Purification of aerolysin

The crude haemolysin sample (20 ml) was filtered through a 0.45 μm syringe filter (Neolus26G, Nerumo, Belgium), and bound to a quarternary aminoethyl (QAE) Sephadex A-50 column (2 × 27 cm, Amersham Biosciences) equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 1M urea. The column was washed with 80 ml of Tris-HCl/Urea buffer and eluted with linear gradient of NaCl from 0.05 to 0.35 M NaCl in 800 ml of the same buffer. The OD280 of the elute was monitored and 2 ml of each fraction from the peak OD280 was collected. The haemolytic activities of each fraction were tested with rabbit erythrocytes (RE).

2.2.2.3 Haemolysis assay

A haemolysis assay was performed as described by Asao et al. (1984). RE, obtained from Moredun Research Institute (MRI), were stored in Alsever's solution at 4 °C. Before use, RE were washed 3 times with the buffer (0.01 M Tris + 0.9% NaCl, pH 7.2) by centrifugation at 500 × g for 5 min at RT and resuspended to a final density of 2×10^6 RE/ml in the same buffer. Seventy microlitre aliquots of aerolysin fractions diluted at 1:3, 1:10, 1:33, 1:100, 1:333, 1:1000, 1:3333, and 1:10000 in PBS were dispensed in duplicate wells of a 96-well tissue culture plate (round bottom, IWAKI, Japan). Thirty microlitre of RE suspension was added to each dilution and incubated in 96-well microplate at RT for 30 min. One haemolytic unit was defined as the activity present in the sample dilution yielding 50% lysis of RE.

Aerolysin samples from the 4 fractions containing strongest activity were pooled and concentrated using Ultrafree—CL Filters (0.1 μm, Millipore). The activity of concentrated aerolysin was again evaluated as described above.

2.2.2.4 Cell treatment with concentrated haemolysin

TLL were harvested 3 days after subculturing and resuspended at 2×10^7 cells/ml in complete IMDM medium. To establish an effective lytic concentration of the haemolysin, 1320 and 2640 Units/ml were evaluated as described by (Goddeeris et al. 1991). Aliquots of 4×10^6 cells (200 μl) were mixed with either 800 μl haemolysin (2640 Units) or 400 μl (1320 Units) in a total volume of 1 ml complete IMDM in FACs tubes (5 ml polypropylene test tube, ELKAY lab products Ltd, UK). Cells were vortexed for several seconds to facilitate lysis and incubated on a rotating mixer for varying periods at RT.

Samples (50 μl) were taken after 10 min, 30 min, and 60 min of aerolysin treatment and the extent lysis was evaluated by flow cytometry (FACS Calibur, Becton Dickinson) after reacting with 2 μg/ml of propidium iodide (PI, a DNA intercalating agent, Sigma) in PBS for 5 min. Data was acquired at 3 min without limitation of the cell number and analysed using FACS Calibur and CellQuest software.

Cell lysis was also evaluated by microscopy of Giemsa stained smears, which allowed assessment of the morphology of aerolysin-treated cells. An aliquot of cell lysis suspension (~3 µl) was taken and loaded onto slides, dried at RT, stained with Giemsa and observed under a light microscope as described as section 2.2.1.2.

2.2.3 Cell treatment with complement

2.2.3.1 Preparation of rabbit serum

The use of antibody and rabbit complement as an alternative means of obtaining lysis of TLL was also tried. Rabbit serum was used as a source of complement. The complement system is composed of more than 25 different proteins and is capable of lysing antibody-coated cells (Janeway et al. 2001). After antibody specifically binds to the cell surface antigen, complement can bind to the complex, initiating a cascade of reactions that leads to the formation of holes in the membrane and results in cell lysis. Ten ml blood samples were taken from each of 5 normal rabbits and stored in 10 ml universal tubes overnight at 4 °C. The sera were then transferred to fresh universal bottles and clarified by centrifugation at 1,730 × *g* (Sorvall® RT7 Plus) for 20 min at RT. Sera were then collected, aliquoted in 5 ml amounts and stored at -20°C.

2.2.3.2 Antibody (ILA88) titration

TLL express MHC class I on their surface and, to determine the saturation binding concentration of ILA88 mAb (Mouse anti-bovine MHC class I monoclonal antibody, IgG2a, International Livestock Research Institute, Kenya), six doubling dilutions from 1:50 to 1:1600 were made in FACS buffer (PBS + 5% FCS + 0.2% NaN_3) in a 96-well round-bottom plate. 100 µl of each dilution was mixed with 100 µl FACS buffer containing 5×10^5 TLL cells in 5 ml FACs tubes. After incubation on ice for 30 min, cells were pelleted by contrifugation at 280 × *g* for 8 min. Cells were then resuspended in 25 µl FACS buffer and mixed with 25 µl goat anti-mouse IgG-Phycoerythrin conjugated (1:300 in FACS buffer, Molecular Probes) and incubated

on ice for 30 min. Cells were washed twice and resuspended in 50 µl PBS before being analysed on the FACS.

2.2.3.3 Complement lysis

Individual sera from 5 rabbits were diluted to 1:2, 1:4, or 1:8 in IMDM medium (without FCS). Two ml TLL at 2×10^7 cells/ml in complete IMDM and 5 µl ILA88 mAb were mixed in a 5 ml tube and incubated on ice for 30 min. After washing twice with IMDM and resuspending in 2 ml IMDM, 2 ml of each diluted serum was added to cells (at final dilutions 1:4, 1:8 and 1:16) and incubated at 37 °C for 60 min. The cells were agitated every 10 min during treatment. The extent of lysis was evaluated by flow cytometry and microscopy as described above.

2.2.4 Purification of *T. lestoquardi* schizonts from cell lysates

To optimise the purification of schizonts, two different gradient centrifugation media, Percoll (1.130 g/ml, Amersham) and Nycodenz (1.310 g/ml, Axis-shield PoC AS) were evaluated.

2.2.4.1 Purification by Percoll gradient centrifugation

The Percoll gradient purification method was carried out essentially as described by Baumgartner et al. (1999). After lysis of ILA88-stained TLL with complement as described above, the cell lysate was centrifuged at $180 \times g$ for 8 min to remove cell debris. The schizont-containing supernatant was collected, centrifuged at 1, $900 \times g$ for 15 min and resuspended in 6 ml complete IMDM medium. One ml of this schizont-enriched supernatant was mixed with 4 ml stock percoll (8.5 parts of Percoll, 0.5 parts of 20 × HEPES, and 1 part 50 mM EDTA, pH7.4) and overlaid with 5 ml 45% Percoll solution in HEPES buffer (10 mM HEPES, 150 mM NaCl, 20 mM KCl, pH7.4) containing 5 mM EDTA in a 10 ml polycarbonate tube (Alpha Ltd, UK). The mixture was centrifuged at 22, $600 \times g$ for 30 min at 4 °C using a SW41 Ti rotor in a Beckman J2-21 ultracentrifuge.

After centrifugation, bands were collected by a Pasteur pipette, transferred to 10 ml HBSS in a 15ml polycarbonate tube (pre-coated with complete medium) and pelleted at 1, 900 × *g* for 10 min. The pellet was washed twice at 1, 900 × *g* for 10 min with HBSS and resuspended in 2 ml complete IMDM medium. Aliquots from each sample were taken for preparation of smears for Giemsa staining to analyse separated schizonts.

2.2.4.2 Purification by Nycodenz gradient centrifugation

Flotation on Nycodenz gradient media has been used for purification *T. parva* schizont from infected cells (Goddeeris et al. 1991). Nycoprep™ (1.310 g/ml, Axis-shield PoC AS), one of several Nycodenz products, was used in the present study. To determine an optimal concentration of Nycodenz gradient for flotation of *T. lestoquardi* schizonts, discontinuous Nycodenz gradients were first made from 25%, 50%, and 75% dilutions in complete IMDM by layering 2ml volumes each, starting with the highest density, in 15 ml polycarbonate tubes.

After cell lysis by staining with ILA88 and complement treatment as described in section 2.2.3.3, 10 ml complete IMDM medium was added to the cell lysates in 15 ml polycarbonate tubes and centrifuged at 180 × *g* for 5 min to pellet cell debris. Two ml supernatant was collected with a pipette and layered onto discontinuous gradients as prepared above in 15 ml tubes. After centrifugation at 3, 360 × *g* (Jouan M4, CR422 centrifuge, UK) for 30 min (no brake), bands from the 3 interfaces were carefully aspirated with Pasteur pipettes, mixed individually with 10 ml complete culture medium in 15 ml polycarbonate tubes and centrifuged at 1900 × *g* for 10 min. The pellets were washed twice at 1900 × *g* for 15 min at RT and resuspended in 1 ml complete IMDM and an aliquot from each sample was taken for smear preparation and Giemsa staining. Preparations were evaluated for schizont yield and purity by examination of Giemsa-stained smears and the dilutions of the gradient steps were refined further as discussed in the results section.

Significant contamination with host cell nuclei was observed in preliminary experiments, suggesting that schizonts and host nuclei have similar densities. To

address this complication, prior to application on the gradients, 10 % (w/v) metrizamide in HBSS was added to the cell lysate according the method described by (Goddeeris et al. 1991) and the mixture incubated for a further 15 min at 37 °C. The capacity of metrizamide to cross nuclear but not schizont membranes allows a differential increase in the density of host-cell nuclei over that of schizonts (Goddeeris et al. 1991).

2.2.5 Analysis of purified schizonts

The purity of schizonts was evaluated first by Giemsa staining as described above and, subsequently, by Trypan blue staining under light microscope, immuno-fluorescence assay and electron microscopy.

2.2.5.1 Trypan blue staining

Trypan blue staining is regarded as vital staining of mammalian cells and other organisms (Allison et al. 1980). Trypan blue stains cells that have lost membrane integrity. Fifty microlitre of 0.4% trypan blue (Sigma) in PBS was mixed with equal volume of schizont suspension in 24-well plate, kept for 10 min at RT, and observed under the microscope using a Neubauer chamber as described above. Live cells exclude the stain while dead cells stain blue. Schizonts cultured overnight were also stained with trypan blue in order to observe their viability under the microscope.

2.2.5.2 Immunofluroscence assay (IFA)

Slides (flow tissue culture multi-test slides –15 well, ICN Ltd) were cleaned in ethanol and allowed to dry. Five microlitre aliquots of purified schizont suspensions were dropped on to each well and fixed in cold methanol for 10 min. Slides were then washed twice in PBS for 10 min. Rabbit serum SA, raised against a mitochondrial heat shock protein 70 of the closely related parasite *T. annulata* (Tamhsp70), kindly provided by Prof. Jabbar Ahmed (Borstel, Germany), was added to the slides at a dilution of 1:200 in PBS and incubated in a humidified chamber for 30 min at RT. Slides were then washed twice in PBS for 5 min. Secondary antibody (FITC-conjugated goat anti-rabbit IgG) at a 1:100 dilution in PBS was then added to the slides and incubated for 30 min at RT. Slides were washed 3 times in PBS for 5

min, mounted in Citifluor and examined using a BX50 fluoresent microscope (Olympus Optical Co Ltd).

2.2.5.3 Electron microscopic analysis of purified schizonts

Electron microscopy was conducted with the assistance of Caroline Goodsir of the Veterinary Laboratory Agency (VLA), UK. Purified schizonts collected from Nycodenz gradients as described above were pelleted by centrifugation at 1900 × g for 15 min and schizont pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH7.3) overnight, washed in dH_2O for 30 min and dehydrated through graded ethanol series comprising 30%, 50%, 70%, 90% and 100% ethanol for 30 min each. The pellets were washed with propylene oxide for 1 hr, then with propylene oxide and araldite (1:1) for 1.5 hrs, and embedded in 100% araldite overnight. Pellets were then embedded in fresh 100% araldite in coffin moulds and incubated at 37 °C for 3 hrs and followed by a further 48 hrs at 60 °C. The blocks were trimmed using a Leica EM Trim and 1 micron sections cut using a Leica Ultra Cut E and glass knife.

Sections were placed on grids, washed in boiled autoclaved dH_2O (BA dH_2O), then in 50% ethanol, for 15 seconds each. The grids were stained by placing on a drop of 2% uranyl acetate for 10 min followed by the addition of a drop of lead citrate for 10 min and washing in NaOH and BA dH_2O for 15 seconds each. Air dried grids were observed under an electron microscope (Jeol 1010) operating at 80 KV.

2.3 Results

2.3.1 Establishment of TLL lines and analysis of cell growth rates

TLL could be observed under the microscope with the appearance of blasts after 7-10 days of infection with *T. lestoquardi* sporozoites. TLL were further examined by Giemsa staining of cytospin smears. TLL of different sizes were observed and, in a significant proportion of individual TLL, more than one schizont was observed (Fig. 2.1).

TLL numbers at different time points after subculture were determined by staining with nigrosin and counting by microscopy and cell numbers were plotted against time to determine the growth curve. Nigrosin enters the cell cytoplasm via the membrane if cells are non-viable and stains them black. Viable cells remain intact and refract light. The cell growth curve indicated exponential expansion between 48 and 72 hrs of culture (Fig. 2.2). It was found that TLL viability decreased after 72 hrs. TLL were sub-cultured every 3-4 days and maintained in the lab.

2.3.2 TLL treatment with aerolysin

2.3.2.1 Activity of purified aerolysin

Aerolysin was extracted from *A. hydrophila* AH-1 and purified on a QAE-50A column. The activity of each fraction was quantified in a RE haemolysis assay. The fractions with the highest aerolysin activity in an initial analysis were pooled and concentrated to a volume of 2 ml. This concentrated sample was then serially diluted and tested for activity. For evaluation of RE lysis, unlysed RE settle to the bottom of the microplate to form a button; completely lysed RE disperse in the solution and no button is seen; incompletely lysis (50% lysis) of RE disperse and also form a button in the bottom. One haemolytic unit was defined as the activity present in the sample dilution yielding 50% lysis of RE. From the activity tested with RE, the final aerolysin dilution capable of lysing 50% of RE was 1:100. Since 30 µl aerolysin was used in the assay, the activity in the stock solution was calculated as 3300 Units/ml.

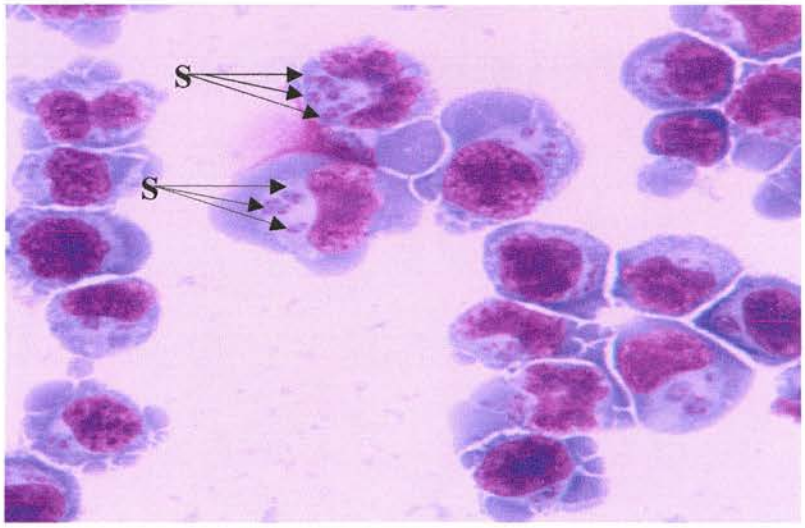


Fig. 2.1 Giemsa stained TLL

Sheep PBMC were infected by *T. Lestoquardi* sporozoites and infected cells were subcultured for 14 days. More than one schizont (S, arrows) was apparent in a significant proportion of TLL. (× 600 magnification)

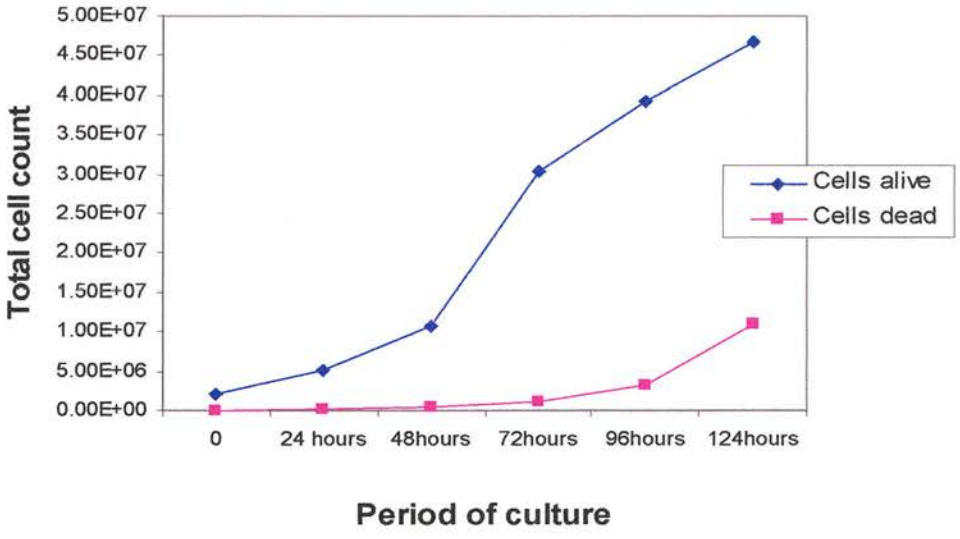


Fig. 2.2 Establishment of cell growth curve.

TLL inoculated at 2×10^5 cells were counted every 24 hrs (24 hrs, 48 hrs, 72 hrs, 96 hrs, and 120 hrs). Numbers of live and dead cells are shown.

2.3.2.2 Treatment of TLL with purified aerolysin

TLL were analysed by FACS after treatment with 1320 and 2640 Units/ml of aerolysin according to the method described by Goddeeris et al. (1991) for 10 min, 30 min, and 60 min. It was observed (Fig. 2.3) that cells treated with 1320 Units/ml of aerolysin had little apparent change in PI uptake and were very similar to the control samples even after 60 min. Some changes were observed after treatment with 2640 Units/ml after 30 min and these were intensified after 60 min incubation.

PI uptake analysis addresses only one aspect of cell status, such as the membrane permeability to PI and subsequent PI binding to host cell DNA. However, the important parameter for this study was cell membrane rupture and release of intracellular schizonts. The integrity of aerolysin-treated cells in this regard may not be reflected in the figures obtained from FACS analysis. To investigate the status of cells after treatment with aerolysin directly, they were stained with Giemsa and observed under the microscope.

2.3.2.3 Giemsa staining of aerolysin-treated TLL

An aliquot of cell suspension treated by aerolysin at 2640 Units/ml for 60 min was dropped onto slides and stained with Giemsa. As shown in Figure 2.4, treated TLL appeared irregular and enlarged. Although there was some evidence of membrane rupture, cells were not completely lysed and schizonts were not released from host cells and remained internal. These observations were in accordance with the FACS analysis and suggested that the aerolysin obtained from *A. hydrophila* AH-1 strain did not adequately lyse TLL.

2.3.3 Cell treatment with complement

2.3.3.1 Titration of ILA88 mAb

TLL were stained with doubling dilutions of ILA88 mAb and analysed by flow cytometry. Saturated staining was achieved with all dilutions between 1:50 and 1:400, with the majority of TLL being negative after treatment with the 1:800

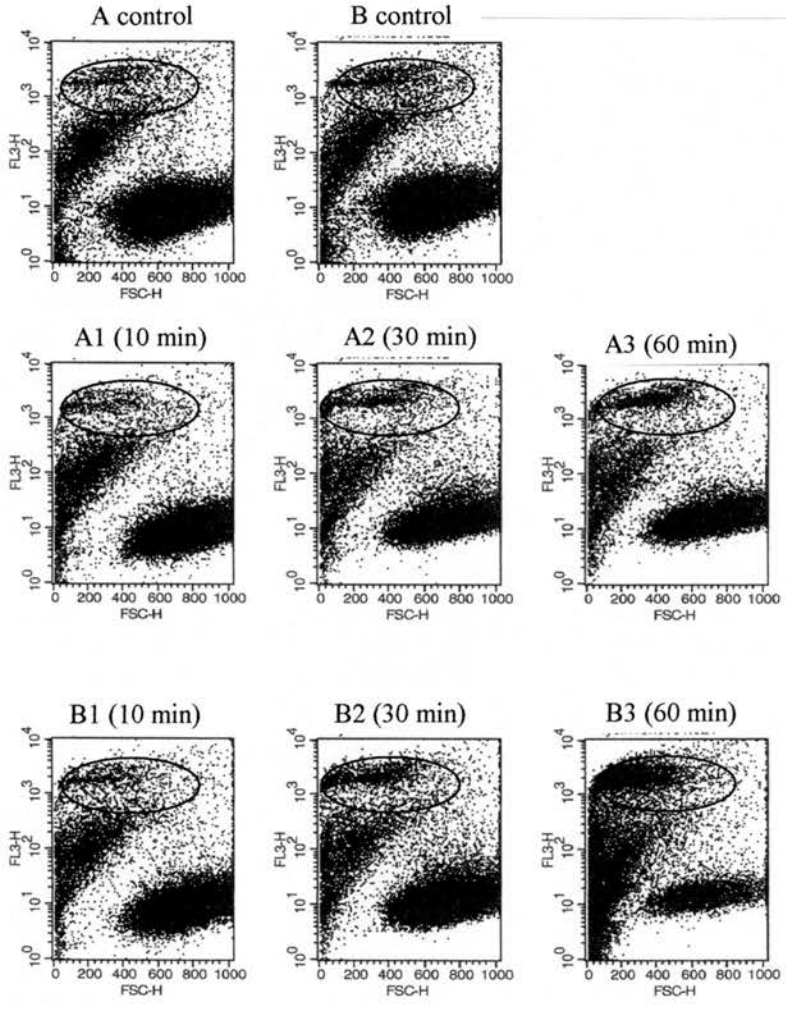


Fig. 2.3 FACs analysis of aerolysin lysis

A-B, TLL controls before treatment (0 min).

A1-3, TLL treated by aerolysin 1320 Units/ml for 10 min (A1), 30 min (A2) and 60 min (A3).

B1-3, TLL treated by aerolysin 2640 Units/ml for 10 min (B1), 30 min (B2) and 60 min (B3).

TLL stained by PI were circled. (FSC-cell size; FL3- PI staining).

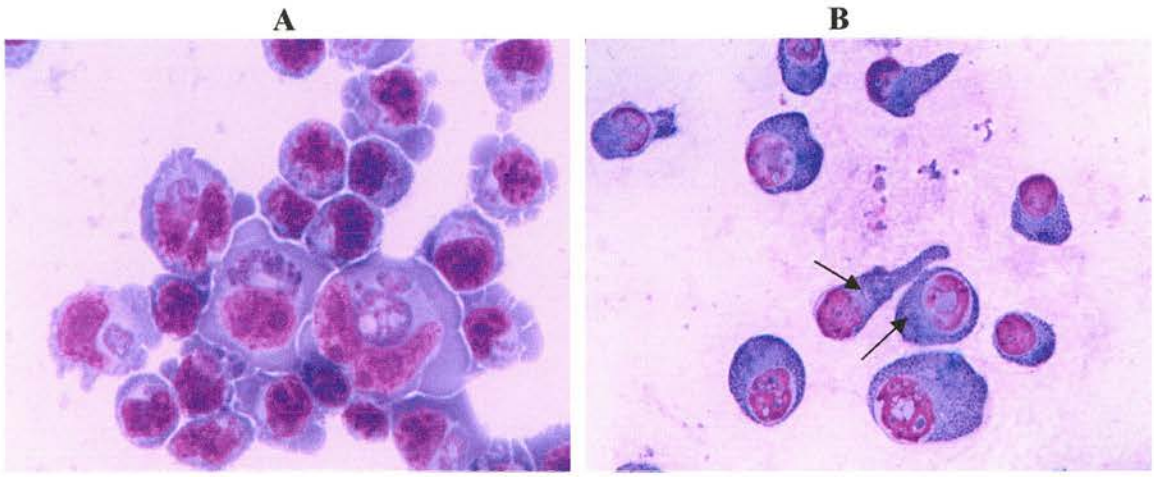


Fig. 2.4. Giemsa stained TLL treated with aerolysin.

Giemsa-stained TLL without treatment (A) and with 2640 Units/ml aerolysin for 60 min (B). The arrow shows schizonts in TLL. (× 400 mag.)

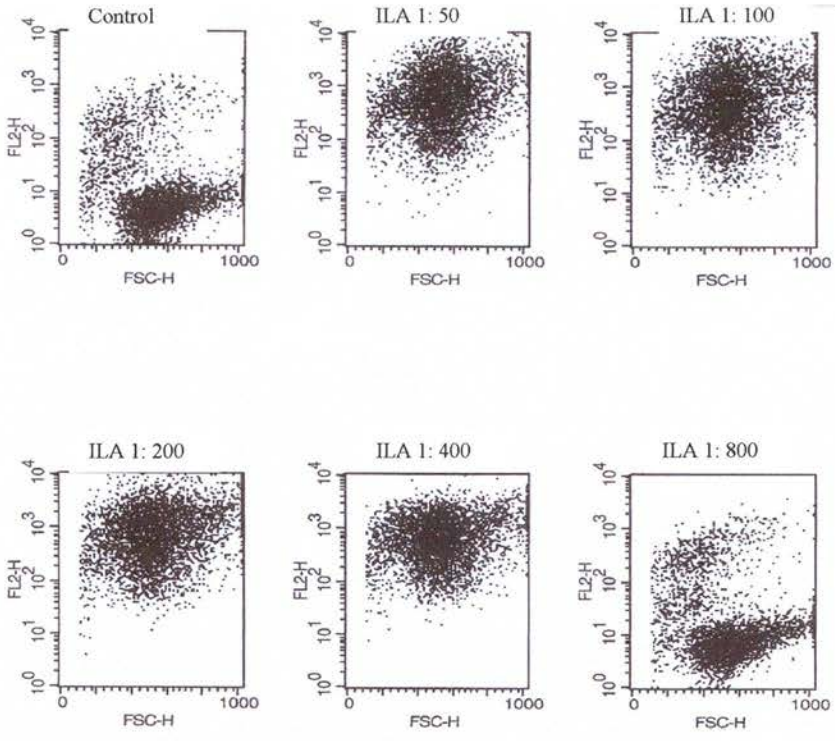


Fig. 2.5 FACs analysis of ILA88 titration

TLL were stained with different dilutions of ILA88 followed by goat anti-mouse IgG-Phycoerythrin (PE) conjugated (1:300 dilution). (FSC-cell size, FL2-ILA88 staining).

dilution (Fig. 2.5). On this basis, the 1:400 dilution was selected for further experiments on complement lysis.

2.3.3.2 Complement lysis of TLL

TLL after staining with ILA88 mAb and complement lysis with pooled rabbit serum revealed that neat serum and the 1:2 dilution gave rise to strong lysis (Fig. 2.6). Lytic activity declined at 1:8 and was almost absent at 1:16. A final dilution of 1:4 was selected as the optimal dilution of rabbit serum for lysing TLL in subsequent experiments.

Analysis of Giemsa-stained smears as prepared from cells treated by complement at 1:4 dilution revealed complete disruption of plasma membranes and loss of the cytoplasmic matrix (Fig. 2.7). Only occasional unlysed host cells were observed (3 - 5%). Schizonts were largely extracellular and retained their morphology.

2.3.4 Purification of schizonts from cell lysates

To obtain pure schizonts free from TLL debris and organelles, two density gradient media, Percoll and Nycodenz, were evaluated.

2.3.4.1 Purification by Percoll gradient centrifugation

After separation of lysed cells on Percoll gradient medium, two thick bands were observed in the upper part of the gradient containing host cell nuclei and debris in addition to a lower band containing schizonts. The upper bands were removed by Pasteur pipette to avoid contamination and the lower schizont-rich band was subsequently collected for preparation of Giemsa staining. From the Giemsa-stained smear, it was found that schizonts were mixed with host cell nuclei and membranes (Fig. 2.8A). It appeared that this method was not suitable for separation schizonts and thereafter Nycodenz gradient medium was tried.

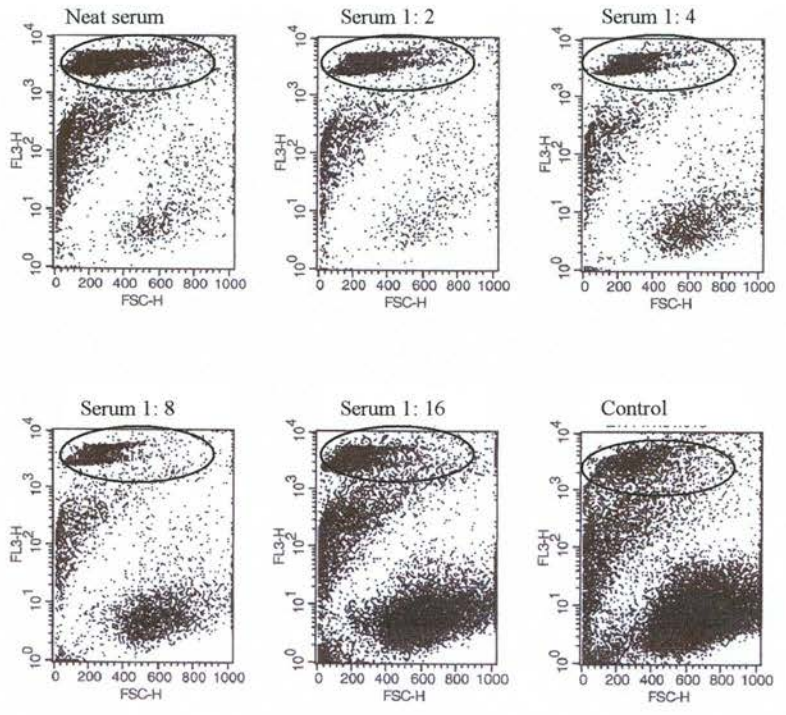
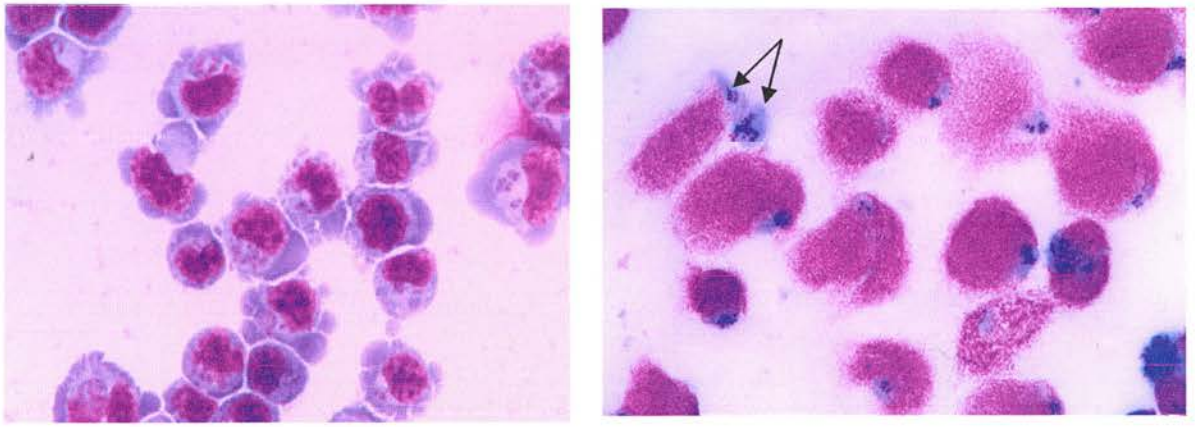


Fig. 2.6. FACs analysis of TLL with complement lysis

TLL were lysed by different dilution of rabbit serum. PI stained cells are circled. (FSC-cell size, FL3- PI staining).



A **B**

Fig. 2.7 Giemsa staining of TLL treated with complement after staining with ILA88 Giemsa-stained TLL with complement (A, 1:4 dilution) or without complement treatment (B). Arrows show released schizonts from TLL. (× 400 mag.)

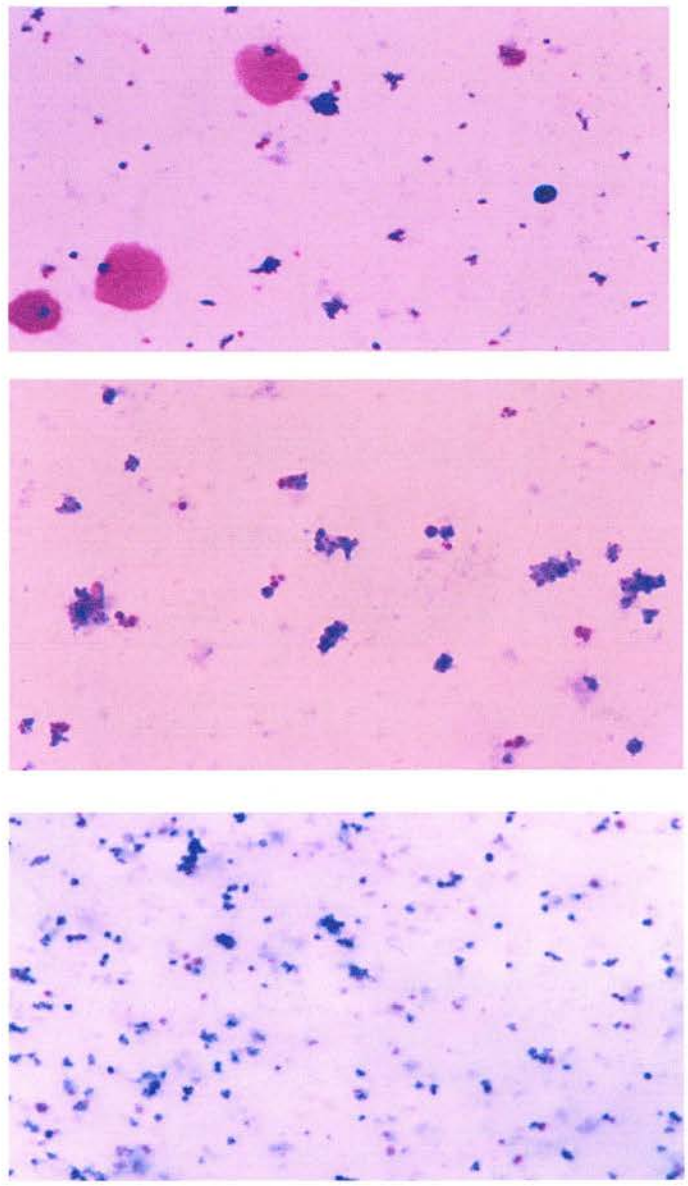


Fig. 2.8 Giemsa-stained purified schizonts

A. Giemsa-stained *T. lestoquardi* schizonts purified with Percoll gradient media. Host cell membranes and nuclei were present. (× 600).

B. Giemsa-stained *T. lestoquardi* schizonts purified with NycoPrep (58% dilution with complete IMDM medium). (× 600).

C. As for B above but with mag. × 400

2.3.4.2 Purification by Nycodenz gradient centrifugation

A three-step discontinuous gradient comprising dilutions of 25%, 50% and 75% Nycodenz media in complete IMDM medium was evaluated in the first instance. After centrifugation of TLL lysate through this gradient, a schizont-enriched band was observed at the interface between 50% and 75%. A Giemsa-stained smear prepared from this band showed enriched schizonts with minimal contamination with host nuclei. To further refine the schizont flotation density, a second gradient comprising dilutions of 50%, 60% and 70% was assessed. This resulted in a schizont-enriched band at the 50% / 60% interface. Finally, a dilution of 58% Nycodenz gradient was found to be optimal for separation of schizonts from cell lysates. The gradient was subsequently used at this concentration as a one-step cushion.

2.3.5 Analysis of purified schizonts

2.3.5.1 Giemsa staining

After flotation on a 58 % Nycodenz cushion, a band was found at the interface between the cell lysate and gradient medium and, when the lysate was pre-treated with 10% metrizamide, intact schizonts were recovered from the band. Isolated schizonts from the band were stained with Giemsa and, from Figure 2.8 B and C, it can be seen to contain pure schizonts in large quantities and free of unlysed cells, cell membranes and nuclei. The purified schizonts retained their intralymphocytic morphology. A pellet was also seen, below the Nycodenz cushion, and was found to consist of cell nuclei, cell debris and some schizonts (data not shown).

This method was selected as the optimal purification of *T. lestoquardi* schizonts from TLL. Schizont yield was estimated by counting Giemsa stained schizonts and it was calculated that up to 8.5×10^6 schizonts could be obtained from 1×10^7 TLL.

2.3.5.2 Trypan blue staining

Separated schizonts were also observed under the microscope by Trypan blue staining in which live schizonts exclude the dye and dead schizonts stain blue. It was

observed that about 96% schizonts exclude blue even after culturing in complete IMDM for overnight (data not shown).

2.3.5.3 Immunofluorescence analysis

Although the purity of schizonts separated from cell lysates was estimated by Giemsa staining (Fig. 2.8 B and C), it might be speculated whether the stained particles were schizonts or derived from other host cell organelles. To confirm this, the *Theileria* parasite specific antiserum SA was used to label purified schizonts. Purified *T. lestoquardi* schizonts were labelled by SA and confirmed to be present in large amounts (Fig. 2.9).

2.3.5.4 Electron microscopic (EM) analysis of separated schizonts

As Giemsa staining and light microscopy does not provide highly resolved detail of purified schizonts, EM was further used to observe separated schizont ultrastructure and membrane integrity. Separated schizonts from cell lysates were seen to be pure under the EM and, in the observed fields, very few host cell mitochondria and other organelles were found (Fig. 2.9). Isolated schizonts from host cells using this protocol were considered viable as the presence of an intact membrane has been regarded as a measure of separated intracellular pathogen viability (Baumgartner et al. 1999).

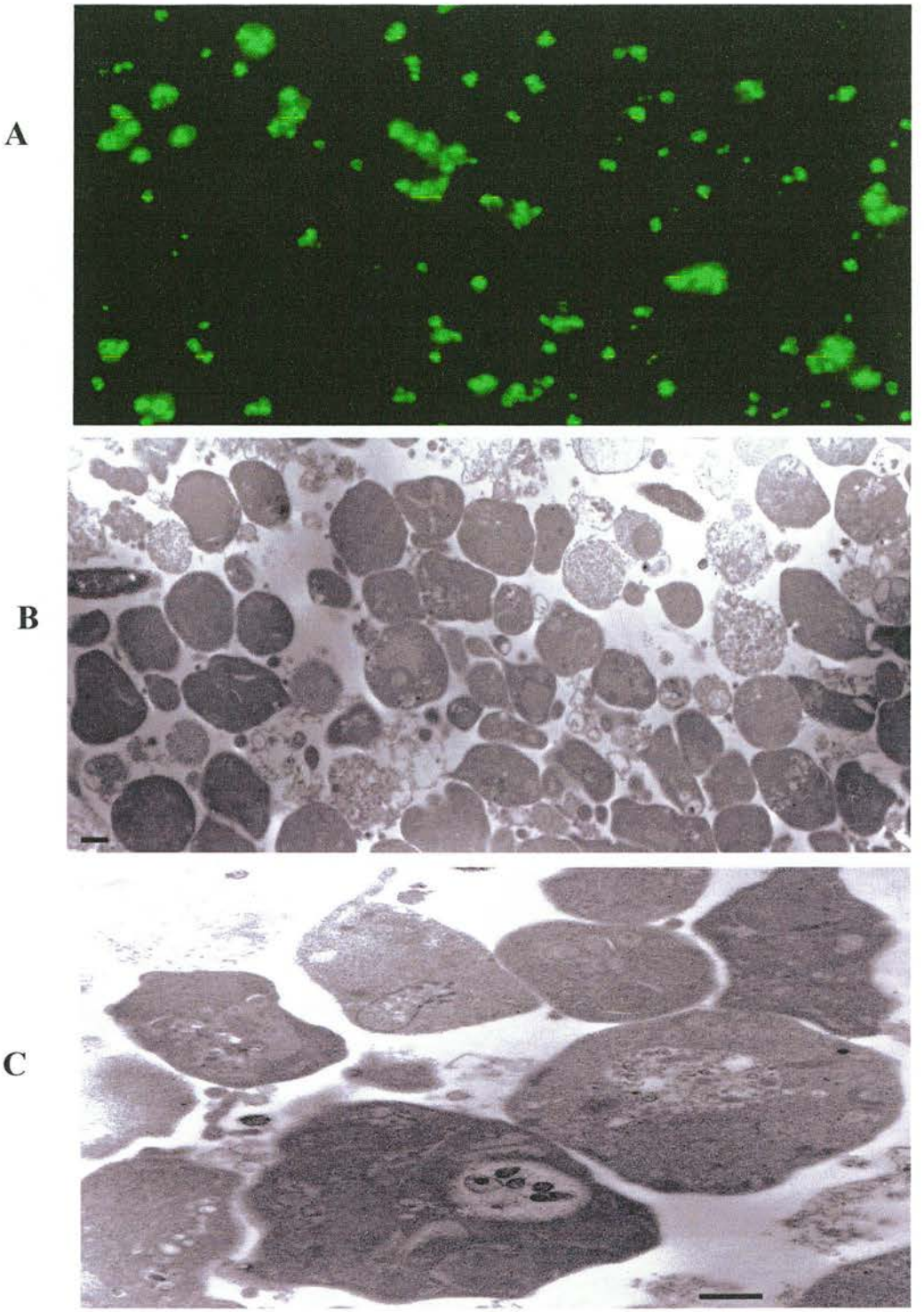


Fig. 2.9 Purified schizonts analysed by IFA and EM

A, Isolated schizonts were labelled by Tamhsp70 Ab (1:200) followed by FITC-conjugated goat anti-rabbit IgG (1:200). (× 400)

B and C, Isolated schizonts were analysed by electron microscopy. bar- 1μm

2.4 Discussion

The present study has resulted in the development of an efficient method for the lysis of TLL and the isolation of pure schizonts from cell lystes.

Separation of schizonts from infected cells requires effective lysis of host cells without incurring damage to the schizont. Aerolysin obtained from *A. hydrophila* was reported to be capable of lysing cells by the formation of discrete transmembrane pores (Asao et al. 1984). Through these pores, small molecules such as amino acids can move in and out, whereas large molecules such as proteins cannot. The cell interior is hyperosmotic, attracting a net influx of water, which results in a sustained cell swelling and subsequent cell lysis (Asao et al. 1984). The effect of aerolysin is confined to mammalian cell membrane and leaves the schizont membrane intact, therefore, schizonts are not affected and viable schizonts can be isolated from cell lysates (Sugimoto et al. 1988). The efficient lysis of *T. parva*-transformed cells by aerolysin has been shown by Sugimoto et al. (1988), Goddeeris et al. (1991) and Baumgartner et al. (1999).

In this study, aerolysin purified from *A. hydrophila* AH-1 was used to treat TLL. It was observed that, under the conditions tested, aerolysin treatment did not lyse TLL and release schizonts efficiently (Fig. 2.3 and 2.4). The failure of aerolysin to lyse TLL may be due to the fact that the AH-1 strain was used in this study as a source of aerolysin as opposed to those of previous workers, who used AH-65 (Sugimoto et al. 1988), which was not available for the present work. A comparison of aerolysin from two strains, AH-1 and AH-65, in the lysis of *T. parva*-infected cells showed that aerolysin obtained from strain AH-65 was more efficient than that derived from strain AH-1 (Sugimoto et al. 1988). In addition, higher concentration and longer incubation was needed for AH-1 to work efficiently.

The complement system is composed of more than 25 different proteins and is capable of lysing antibody-coated cells (Janeway et al. 2001). Complement has been successfully used for lysis of malaria parasite-infected erythrocytes and it was reported that complement treatment broke the erythrocyte membrane and left the intracellular

malaria parasite membrane intact (Trager et al. 1972). In the present study, complement from rabbit serum efficiently lysed ILA88-stained TLL and allowed release of schizonts. Released schizonts from complement lysed TLL were intact and retained their morphology (Fig. 2.7). In addition, using mechanical agitation for 3-5 times (section 2.2.3.3), schizonts can be physically separated from host cell cytoplasm. This suggests that complement treatment with mechanical agitation is an efficient method for lysing TLL.

To purify schizonts after TLL lysis, it is necessary to isolate them from cell lysates. To obtain pure *T. parva* schizonts free from other cell components, Sugimoto et al. (1988), Goddeeris et al. (1991) and Baumgartner et al. (1999) used different gradients and found them to be suitable for schizont purification.

In the present study, two different gradient media, Percoll and Nycodenz, were investigated. It was found schizonts derived from Percoll gradient medium contained unlysed host cells and nuclei (Fig. 2.8A). Percoll gradient medium has been used for purifying *T. parva* schizonts and small amounts of unlysed host cells and nuclei were also observed (Sugimoto et al. 1988). In addition, as it requires high *g* -force centrifugation (22, 600 × *g*), it may cause parasite membrane damage and, therefore, affect schizont viability. This method is possibly more suitable for DNA or RNA extraction from purified schizonts than for producing schizonts for culture and identification of secreted proteins.

Nycodenz is an alternative gradient used for subcellular organelle separation and has several advantages compared to Percoll gradient. It can be easily removed from samples after the fractionation is completed; it is not precipitated at low pH and samples can be isolated from gradient fractions by acid precipitation; it is much less toxic to cells than Percoll and cells exposed to Nycodenz retain a more normal morphology (Mutzel et al. 1980; Rickwood et al. 1982). Nycodenz gradient media has also been used for isolation of *T. parva* schizonts (Goddeeris et al. 1991) and *T. gondii* sachyzoites (Coppens et al. 2000). Furthermore, it requires much lower *g* × force centrifugation (3, 360 × *g*) compared to Percoll gradient media. In this regard,

NycoPrep media may be more suitable for producing schizonts for culture. After evaluation of a broad range of concentrations, 58% (v/v) NycoPrep dilution was shown to be optimal for flotation of schizonts.

The nucleus of the host cell has been found to have a similar size and density as the schizont and it is therefore difficult to separate each from the other (Goddeeris et al. 1991). In the present study, 10% metrizamide was used to increase host cell density in TLL and, after centrifugation with 58% Nycodenz gradient, it was observed that host nuclei were efficiently separated from schizonts (Fig. 2.8 B and C).

The purity of isolated schizonts was considered the most important factor for evaluating schizont quality in this study. Using 58% Nycodenz gradient, schizont purity was tested by Giemsa staining (Fig. 2.8 B and C) and EM analysis (Fig. 2.9 B and C). It was observed that large numbers of intact schizonts were obtained that were free of unlysed TLL and cell nuclei. This suggests that schizonts isolated by the method have a high level of purity. Although some cell debris and few mitochondria were observed by EM, contamination was estimated to be minimal.

Goddeeris et al. (1991) expressed schizont yield in terms of schizont numbers as a percentage of quantity of cells used as a source, and reported yields as high as 84%. In the present study, Giemsa staining showed that approximately 8.5×10^6 schizonts could be obtained from 1×10^7 TLL. However, this is unlikely to reflect an 85% yield since a proportion of TLL contain multiple schizonts (section 2.3.1). In this regard, Hulliger (1965) reported that *T. parva* and *T. annulata* transformed cells contain multiple schizonts in each infected cell.

Viability is another important factor for evaluating isolated schizonts especially when schizonts are required for *in vitro* culturing. Trypan blue staining is regarded as vital staining with viable cells excluding the dye and dead cells staining blue (Allison et al. 1980). Trypan blue was used to evaluate purified schizont viability in this study. It was found that schizonts recovered using the above methods excluded the dye and the majority (96%) remained viable after culturing overnight in complete

IMDM. This would provide adequate time for schizonts to secrete antigens into the supernatant and allow identification of schizont secreted proteins. In the study of Baumgartner et al. (1999), purified schizonts were regarded as viable when the schizont membrane was seen to be intact by EM analysis. In the present study, EM analysis showed that schizonts separated from cell lysates had ultrastructural integrity and intact membranes (Fig. 2.9 B and C), providing further evidence that the purification method yielded viable schizonts.

The viability of purified *P. falciparum* parasites from infected erythrocytes was tested on the basis of their ability to infect erythrocytes and complete schizogony *in vitro* (Pavia et al. 1983). However, from *Theileria* parasite life cycle, the schizonts in infected host cells require further differentiation to merozoites to enable them to invade erythrocytes (Jura et al. 1983). Whether isolated schizonts can infect host leucocytes in vitro remains to be determined and needs to be investigated in future. Should it be the case, it would prove a more stringent evaluation of schizont viability.

In summary, an efficient method for the lysis and purification of *T. lestoquardi* schizonts from infected cells was established. Complement treatment of *T. lestoquardi*-infected cells was effective in releasing schizonts from host cell cytoplasm. Released schizonts were intact and retained their morphology. An optimal dilution of Nycoprep gradient medium was effective for separation of schizonts from cell components. Purified viable schizonts were obtained with high yields and could be maintained *in vitro* overnight. The method was therefore considered appropriate for further experiments aimed at identification of antigens secreted by the schizont.

Chapter 3: Optimised metabolic labelling of *T. lestoquardi*-infected cells

3.1 Introduction

The aim of this study was to identify proteins secreted by *T. lestoquardi* schizonts to assist in the characterisation of host-parasite interactions. At the outset of the study, limited information was available regarding *Theileria* secreted proteins, and this may have been due partly to the difficulty of obtaining starting materials of schizonts in high yield and of sufficient purity. The establishment of a method for isolation of *T. lestoquardi* schizonts and their short-term culture described in the previous chapter provided an opportunity for collecting and analysing proteins elaborated by the parasite into the culture supernatant. However, this approach posed two problems. Firstly, because maintenance of parasite viability in overnight culture required the presence of FCS, culture supernatants would contain large amounts of host-derived proteins. Secondly, the inevitably small amounts of parasite proteins elaborated into the culture would be difficult to detect in SDS-PAGE gels stained by conventional methods such as Coomassie blue or silver.

A possible solution to these difficulties would be to metabolically label infected cells prior to isolation of schizonts. This would allow the identification of parasite proteins in culture supernatants by SDS-PAGE and autoradiography. Metabolic radio-labelling of infected cells with ^{35}S methionine has been employed previously for the detection of changes in protein expression of bovine lymphocytes induced by *T. parva* infection. In that study, a number of proteins were identified in infected cells by 2D-electrophoresis that were not present in un-infected cells or in isolated *T. parva* schizonts (Sugimoto et al. 1988; 1991).

The present study involved adaptation of this methodology to provide metabolically labelled schizonts for axenic culture and identification of putative secreted proteins.

3.2 Materials and methods

To ensure adequate sensitivity for detection of radio-labelled schizont proteins after separation, a number of parameters were evaluated and optimised. These comprised: 1) duration of the labelling period; 2) cell density in the labelling mix; and 3) quantity of radioactivity added.

3.2.1 Duration of the labelling period

TLL in exponential growth phase were harvested and centrifuged at 280 × *g* for 10 min, washed twice in PBS and resuspended at a density of 1×10^6 cells/ml in methionine/cysteine-free Dulbecco's Modified Eagle's medium (MCF-DMEM, Sigma) supplemented with 2 mM L-glutamine and 5% dialyzed FCS, in which methionine and cysteine are absent. A 14 µl aliquot of ^{35}S-labelled methionine/cysteine (14.29 µCi/µl, PRO-MIX, Amersham Pharmacia Biotech Ltd) was added to 500 µl of MCF-DMEM and divided between two T 25 cm^2 tissue culture flasks, each containing 5 ml of cell suspension and giving a final ^{35}S methionine/cysteine concentration of 20 µCi/ml. The flasks were then incubated at 37 °C in 5% CO_2 incubator, one for 6 hrs and the other for 18 hrs.

After labelling, cells were harvested, washed twice with PBS, and resuspended in 500 µl PBS. ^{35}S isotope incorporation by TLL was measured by adding 5 µl of cell suspension to 2 ml scintillant cocktails (Universal Lsc-Cocktail, Beckman, UK) in triplicate and counting in a Liquid Scintillation Analyzer (2500 TR, Beckman, UK). Schizonts were separated from 5×10^6 radio-labelled TLL using the method described in section 2.2.4 and resuspended in 1 ml of complete IMDM. Incorporation of radioactivity in schizonts was measured by scintillation counting as above.

3.2.2 Optimal labelling cell density

To determine the maximum radio-labelling cell density consistent with adequate viability, a range of cell densities from 2×10^6 cells/ml to 64×10^6 cells/ml in doubling dilutions was examined after 18 hrs subculture. Aliquots (1.0 ml) of cells at different densities were cultured overnight in 24-well cell culture plates (Costar, US)

and the percentage cell viability was determined by staining with Nigrosin as described in section 2.2.1.

Once the range of cell densities consistent with high viability was determined, the influence of cell density on radioisotope incorporation was evaluated. TLL were seeded in 1.0 ml aliquots in 24 well plates at densities from 1×10^6 to 8×10^6 cells/ml in doubling dilutions and cells were labelled with 20 μCi/ml ^{35}S methionine/cysteine for 18 hrs at 37 °C. Cells were harvested, washed and resuspended in 50 μl PBS, and ^{35}S uptake was measured by liquid scintillation as described above.

The extent of ^{35}S incorporation by cellular proteins was analysed by SDS-PAGE (Laemmli 1970) followed by autoradiography. Aliquots of 10 μl cell suspension were mixed with an equal volume of Laemmli sample buffer (62.5 mM Tris, pH6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue), boiled for 5 min, and loaded onto a 4% /12% discontinuous SDS-PAGE mini-gel (Bio-Rad). Electrophoresis running buffer consisted of 25 mM Tris, 192 mM glycine and 1.3 mM SDS (pH8.3). Proteins were separated at 200V for 50 min using a Bio-Rad Protean II apparatus (Bio-Rad). Separated proteins were visualized by staining the gel with 0.1% Coomassie Blue R250 (Sigma) in dH_2O/10% glacial acetic acid/50% methanol for 30 min, followed by destaining in dH_2O/10% glacial acetic acid/25% methanol for 45 min. For autoradiography, gels were dried under vacuum at 80 °C using a GD2000 Hoefer Slab Gel Drier (Amersham Biosciences) and exposed to Fuji RX-100 film (Fuji Film Co., Japan) for 3-5 days at –20 °C.

3.2.3 Influence of radioisotope quantity on uptake of labelling

To maximise radio-labelling of TLL, different quantities of ^{35}S isotope were evaluated. TLL (2×10^6 cells/ml) in 5 ml of MCF-DMEM medium were incubated with 20 or 40 μCi/ml of ^{35}S methoinine/cysteine in 25 cm^2 tissue culture flasks. Cells were harvested after 18 hrs, washed with IMDM and resuspended in 500 μl PBS. A

10 µl aliquot was taken from each flask, analysed by scintillation counting and SDS-PAGE and autoradiography as described above.

Schizonts were separated from 1×10^7 radio-labelled TLL as described in section 2.2.4 and resuspended in 50 µl PBS. The level of ^{35}S incorporation in schizonts was evaluated by scintillation counting and autoradiography of SDS-PAGE as described above.

3.2.4 Detection of labelled soluble schizont proteins present in culture supernatant

Schizonts were isolated from 1×10^7 metabolically labelled TLL as described above and resuspended in 4 ml complete IMDM. Schizont suspensions were then incubated in wells of 24-well plates (1ml/well) at 37 °C for 6 hrs or 18 hrs (2 wells each). Schizont cultures were then centrifuged at $2500 \times g$ for 20 min and the supernatant was collected and concentrated 10-fold using a Tm10 ultrafiltration cell (5 kDa cut off, Amicon). The presence of schizont proteins in the supernatant was then evaluated by SDS-PAGE followed by autoradiography. A 10 µl aliquot of concentrated supernatant was mixed with an equal volume of Laemmli sample buffer and loaded onto an SDS-PAGE gel as described above. Labelled protein bands were then detected by SDS-PAGE and autoradiography as described in section 3.2.2.

3.2.5 Secretion inhibition analysis

To evaluate whether schizont proteins in culture supernatants arose through secretion, supernatants were examined after culture at 4 °C, or at 37 °C in the presence or absence of secretory inhibitors. In the first of these experiments, labelled schizonts isolated from 1×10^7 TLL were resuspended in 1 ml complete IMDM and divided between two tubes. One sample was cultured at 4 °C and the other at 37 °C overnight. Supernatants from the two cultures were then collected and analysed by SDS-PAGE followed by autoradiography.

In the second experiment, two secretory inhibitors, Brefeldin A (BFA) and monensin, were used. Monensin is a Na+ ionophore that prevents glycoprotein

secretion (Schuerwegh et al. 2001) and BFA, a fungal metabolite, is a carboxylated ionophore that blocks protein secretion by inhibiting the formation of transfer vesicles that move proteins from the ER to the Golgi apparatus (Klausner et al. 1992). Purified schizonts isolated from 3×10^7 TLL radio-labelled with ^{35}S methionine/cysteine as described previously were resuspended in 4 ml complete IMDM and divided into four wells (1.0 ml/each well) of a 24-well culture plate. Two wells were cultured with 10 μg/ml BFA (made from 1mg/ml stock solution in DMSO, Sigma) and 20 μM monensin (made from 1mM Monensin stock solution in DMSO, Sigma), respectively. Two wells of controls were cultured in DMEM without inhibitors. Schizonts were then incubated at 37 °C for 18 hrs. Supernatants were collected and concentrated as described above. Concentrated supernatant (10 μl) from each sample was mixed with an equal volume of Laemmli sample buffer and analysed by SDS-PAGE and autoradiography.

3.2.6 Immunoblot analysis of known secreted proteins

To determine whether schizont culture supernatants contain known secreted proteins of *Theileria* schizonts, antisera raised against the *T. annulata* schizont DNA binding protein, SuAT (Shiels et al. 2004) and mitochondrial hsp70 (Tamhsp70, Schnittger et al. 2000) were obtained from Dr Brian Shiels, UK and Prof. Jabbar Ahmed, Germany, respectively. Both proteins have been shown to be secreted by *T. annulata* schizonts (Schnittger et al. 2000; Shiels B et al., 2004) and, since *T. annulata* and *T.lestoquardi* are closely related species (Kirvar et al. 1998; Leemans et al. 1999b), it was considered that the sera would recognize the *T. lestoquardi* protein homologues.

Isolated schizonts from 2×10^7 TLL and culture supernatants were analysed by SDS-PAGE as described above. Briefly, concentrated supernatant samples (10 μl) were mixed with an equal volume of Laemmli sample buffer, loaded to gels along with standard protein markers (5μl per track, prestained, BioRad) and separated by SDS-PAGE. After electrophoresis, separated schizont proteins were transferred to a nitrocellulose membrane according to the method described by Dunn (Dunn 1986). The gel, filter papers (3 mm, Whatman) and nitrocellulose (Hyperbond, Amersham) were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 25% methanol, pH

9.2) for 5 min. Two sheets of filter paper were then placed on the anode plate of an Immuno-blotter (Bio-Rad), followed by the nitrocellulose membrane, the gel, and two additional pieces of filter paper. Air bubbles were removed by rolling a pipette over the stack. The cathode plate assembly was then fitted and proteins were electro-transferred at 300 mA for 45 min.

After transfer, the nitrocellulose was incubated in 50 ml blocking buffer (5% milk powder in PBS/0.05%Tween80) for 30 min. 10 ml of either SuAT or SA rabbit sera, or the appropriate pre-immune serum diluted to 1:200 in blocking buffer were then added and the blot was incubated for 1 hr at RT on a rotating platform. The nitrocellulose was then washed 3 times for 5 min with washing buffer (PBS/0.05%Tween80), and 10 ml horse radish peroxidase (HRP)-conjucted goat anti-rabbit IgG (DAKO), at a dilution of 1:1000 in blocking buffer, was added. The membrane was shaken for a further 1 hr. After a further three 5 min washes in washing buffer, 10 ml developer solution (0.1mg/ml DAB in 0.05% H_2O_2 in washing buffer) was added and allowed to react for 15-20 min until adequately developed. The membrane was then rinsed in dH_2O to stop the reaction.

3.3 Results

3.3.1 Duration of the labelling period

To evaluate the influence of the duration of the labelling period, TLL were incubated in the labelling mix for 6 hrs or 18 hrs and examined for incorporation of radioactivity. It was observed that only low levels of activity were incorporated after 6 hrs, with only 3.41% of total activity being taken up by TLL, of which only negligible amounts were detected in the schizont fraction (Table 3.1). In contrast, incubation for 18 hrs gave rise to greater uptake of radio-label by both host cells and intracellular schizonts (13.21% and 3.92% of the radioactivity, respectively), with over 25% of incorporated label being located in the schizont fraction.

	Duration of culture	
	6 hrs	18 hrs
Schizonts	0.01(0.005%)	7.84 (3.92%)
TLL	6.81(3.41%)	26.42 (13.21%)

Table 3.1 Incorporation of ^{35}S ($\times 10^6$ cpm) by TLL and schizonts after labelling for 6 and 18 hrs. Figures indicate ^{35}S incorporation with cpm from 5×10^6 cells or their schizont yield. Values in parentheses indicate percentage of total radioactivity incorporation in the labelling reaction.

3.3.2 Influence of cell density on metabolic labelling

To determine maximum cell density for overnight culture consistent with adequate viability for radio-labelling, TLL were seeded in 24-well plates at densities ranging from 2×10^6 to 64×10^6 cells/ml and assessed for viability after 18 hrs culture. Cell viability was seen to decrease with increasing cell density, with 94% of cells being viable at 2×10^6 cells/ml, 89% at 4×10^6 cells/ml, 78% at 8×10^6 cells/ml and 52% at 16×10^6 cells/ml (Fig. 3.1). Based on these observations, incorporation of radio-

labelling was evaluated at seeding densities ranging from 1×10^6 cells/ml to 8×10^6 cells/ml.

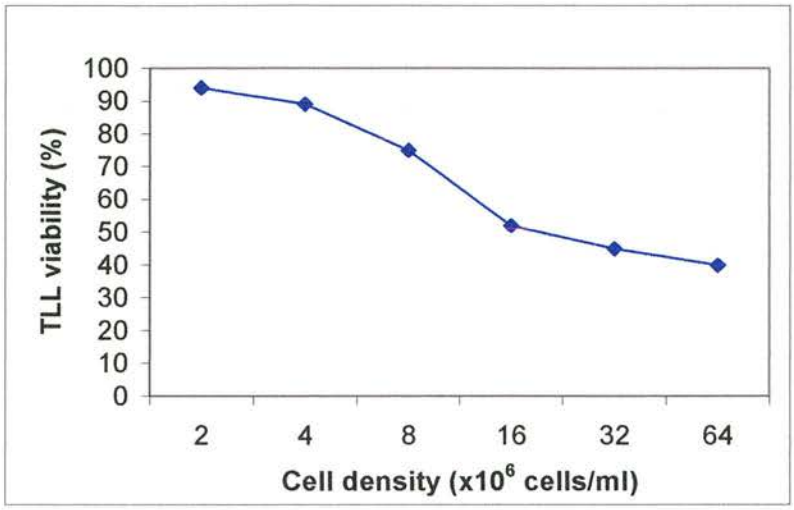


Fig. 3.1 Measurement of TLL viability at different densities

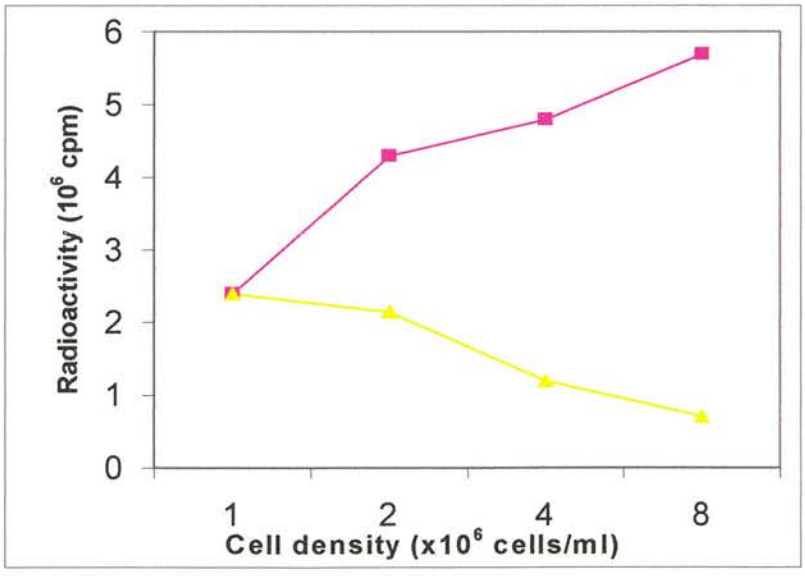


Fig. 3.2 ^{35}S incorporation by TLL seeded at different densities. Total incorporation by the culture (—■—) and activity incorporated per cell (—▲—) are shown.

It was observed that, although the total amount of labelling increased with increasing seeding density, the activity associated with each cell declined as density was increased from 1×10^6 to 8×10^6 cells/ml (Fig. 3.2).

In addition, examination of a SDS-PAGE autoradiograph prepared from labelled cells revealed that, with equivalent cell loading, protein bands isolated from cells seeded at lower densities were more intense (Fig. 3.3). This was in agreement with the results obtained from scintillation counting. Since the optimal growth of the cells was obtained at a density of 2×10^6 cells/ml and efficient incorporation of ^{35}S methionine/cysteine was also obtained at this density, radio-labelling was carried out at this cell density in subsequent experiments.

3.3.3 Incorporation of ^{35}S methionine/cysteine with increasing radioactivity

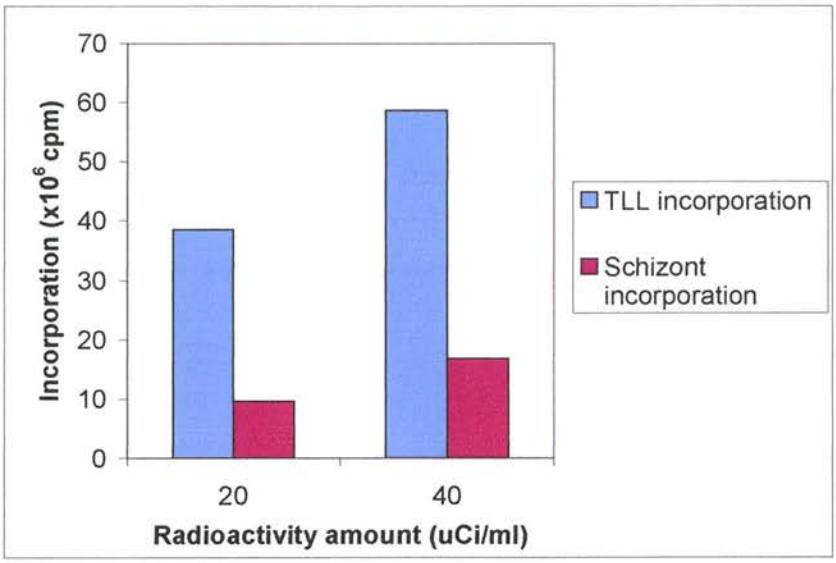


Fig. 3.4 Radioactivity of ^{35}S at 20 and 40 μCi/ml incorporated into TLL and schizonts

Having established optimal incubation time and cell density, the effect of increased concentration of radio-label in the culture on the level of incorporation by TLL was examined. Cultures were labelled with 20 μCi/ml (Sugimato et al. 1991) and 40 μCi/ml of ^{35}S methionine/cysteine, and the amount of incorporated label was

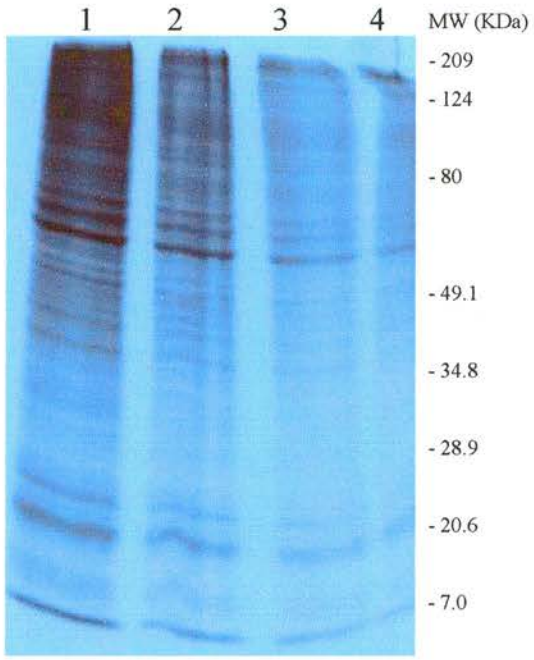


Fig. 3.3 SDS-PAGE autoradiograph of TLL labelled with ^{35}S methionine/cysteine for 18 hrs. The equivalent of 5×10^5 cells was loaded in each lane. Lane 1, 1×10^6 cells/ml; Lane 2, 2×10^6 cells/ml; Lane 3, 4×10^6 cells/ml; Lane 4, 8×10^6 cells/ml.

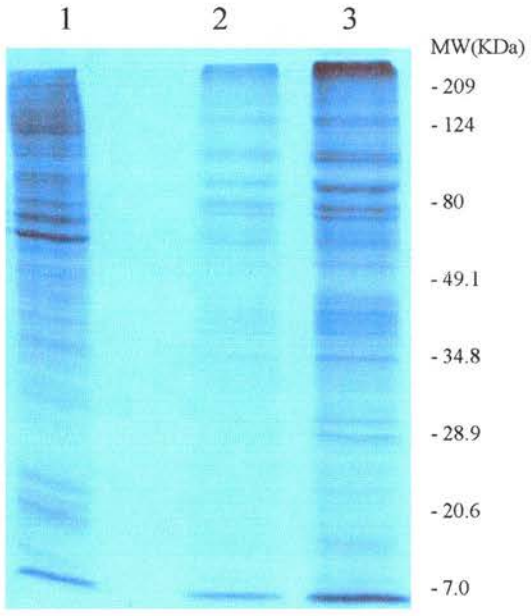


Fig. 3.5 SDS-PAGE autoradiograph of TLL and purified schizonts

Schizonts were purified from TLL labeled with ^{35}S methionine/cysteine.

Lane 1, 5×10^5 TLL; Lane 2, schizonts from 5×10^5 TLL; Lane 3, schizonts from 2×10^6 TLL.

compared by scintillation counting. It was observed that, when the amount of added isotope was increased from 20 to 40 μCi/ml, total cellular incorporation increased from 38.5×10^6 cpm to 58.6×10^6 cpm. Intracellular schizont incorporation also increased, from 9.62×10^6 cpm to 14.84×10^6 cpm. Subsequent labelling experiments were therefore conducted using 40 μCi/ml ^{35}S methionine/cysteine.

Radio-labelled TLL and separated schizonts were visualized by SDS-PAGE followed by autoradiography. It was observed that proteins from TLL seeded at 2×10^6 cells/ml and schizonts derived from them were efficiently labelled with 40 μCi/ml ^{35}S methionine/cysteine after incubation for 18 hrs (Fig. 3.5). Over 30 protein bands were observed in samples taken from labelled schizonts. The dominant schizont protein bands were approximately 92 kDa, 70 kDa, 65 kDa, 60 kDa, 52 kDa, 41 kDa, 35 kDa and 31 kDa in size, whereas major proteins from TLL were approximately 130 kDa, 120 kDa, 115 kDa, 95 kDa, 86 kDa, 72 kDa, 63 kDa, 55 kDa, 48 kDa, 35 kDa and 25 kDa. The radio-labelled protein patterns from TLL and isolated schizont appeared distinct, with several dominant protein species being peculiar to either TLL or schizont fractions.

3.3.4 SDS-PAGE analysis of secreted proteins labelled with ^{35}S

Schizonts were cultured for 6 hrs and 18 hrs in complete IMDM after separation of schizonts from labelled TLL as described above. The supernatants were then collected and analysed by SDS-PAGE followed by autoradiography. No apparent ^{35}S-labelled proteins were detectable after 6 hrs culture. However, several bands were seen after 18 hrs incubation, ranging in size from 20 kDa to 150 kDa, with abundant species of 100 kDa, 70 kDa and 60 kDa (Fig. 3.6).

3.3.5 Schizont secretion inhibition analysis

To evaluate whether labelled proteins in schizont culture supernatants arose through secretion, supernatants from metabolically labelled schizonts cultured for 18 hrs at 4 °C and 37 °C were compared by SDS-PAGE and autoradiography. No protein bands were observed in supernatants derived from 4 °C cultures (Fig. 3.7). In contrast, multiple protein species were observed in material from 37 °C cultures (Fig.

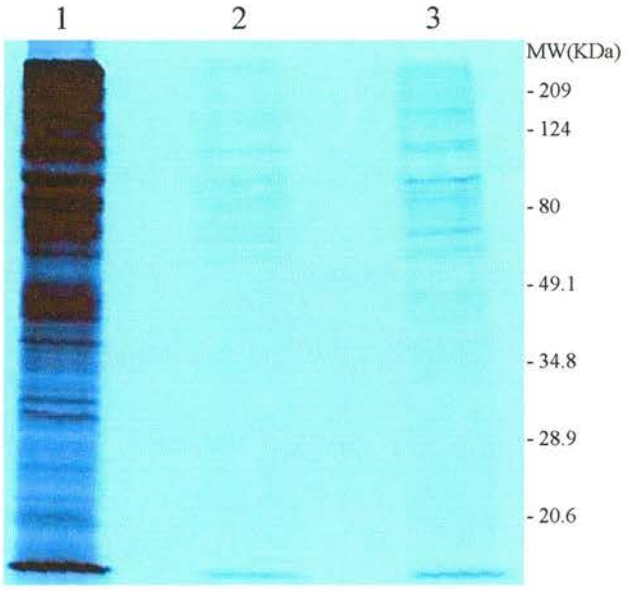


Fig. 3.6 SDS-PAGE autoradiograph of *T. lestoquardi* schizonts and secreted proteins Lane 1, schizonts isolated from 5×10^6 TLL. Lane 2, supernatant of 6 hrs culture in complete IMDM. Lane 3, supernatant of 18 hrs culture in complete IMDM.

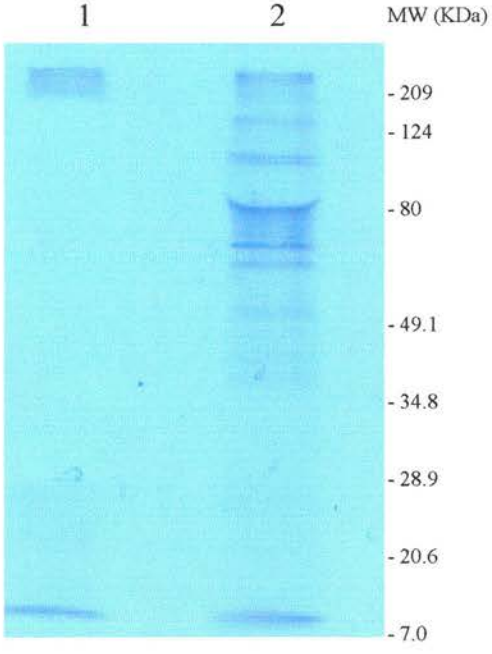


Fig. 3.7 SDS-PAGE autoradiography of supernatants from *T. lestoquardi* schizonts Schizonts derived from 2×10^6 TLL were cultured for 18 hrs at 4 °C (lane 1) and 37 °C (lane 2), respectively.

3.7), providing evidence that metabolic activity is required for elaboration of labelled proteins into the culture medium.

To further examine the mechanisms responsible for elaboration of schizont proteins into the culture medium, labelled schizonts were cultured for 18 hrs in the presence of the secretory inhibitors, BFA or monensin. It was observed that, with equivalent loading in SDS-PAGE analysis, the amounts of ^{35}S-labelled proteins appeared somewhat reduced in supernatant from BFA-treated schizonts, while no apparent difference was observed in monensin-treated schizont supernatants when compared with untreated schizonts (Fig. 3.8). In addition, in BFA-treated schizont supernatant, two bands of 50 kDa and 35 kDa appeared reduced, and two bands of 90 kDa and 60 kDa were seen increased when compared with untreated cultures, although the total number of protein bands seemed unaffected by the treatment (Fig. 3.8).

3.3.6 Immunoblot analysis

To verify that supernatants of schizont cultures contained known secreted proteins, immunoblot analysis was conducted using rabbit sera raised against the SuAT DNA binding protein and the mitochondrial hsp70 of *T. annulata*. The SuAT antiserum showed strong recognition of a 66 kDa band and weakly recognised an additional band at 110 kDa (Fig. 3.9). These observations are consistent with the specificity of this antiserum for *T. annulata* (Shiels B. personal communication). The serum raised against *T. annulata* mitochondrial hsp70 recognised a distinct band of 70 kDa in schizont culture supernatants (Fig. 3.10). No reactivity was observed with control rabbit sera.

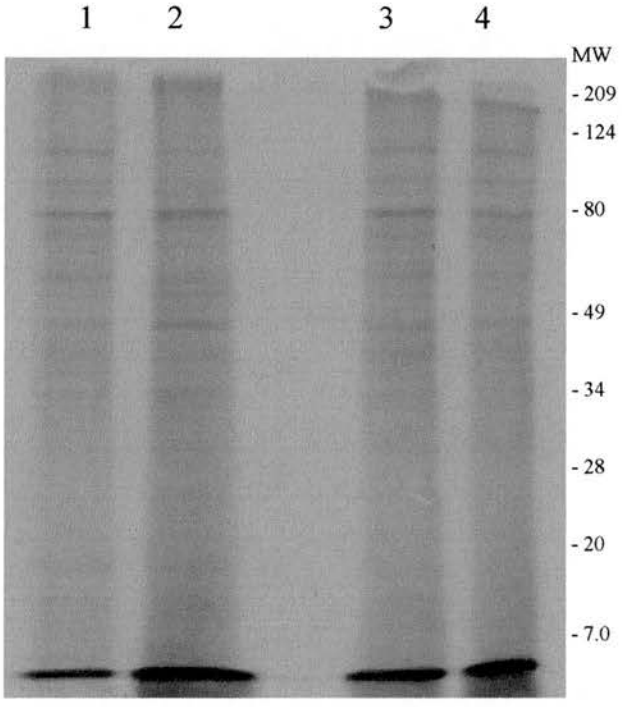


Fig. 3.8 Effect of secretory inhibitors on elaboration of schizont proteins into culture supernatants.

Equal volumes of culture supernatants were loaded in each lane.

Lane 1, supernatant from radio-labelled schizont culture treated with BFA (10 μg/ml).

Lane4, supernatant from radio-labelled schizont culture treated with monensin (20 μM).

Lane 2 and lane 3, supernatants of untreated schizont culture.

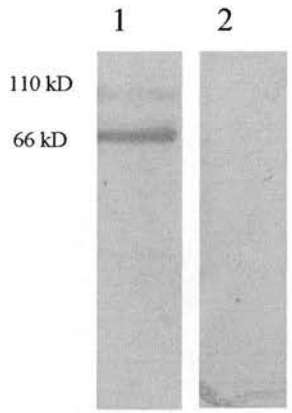


Fig. 3.9 Immuno-blot of SDS-PAGE separated schizont culture supernatant probed with SuAT-specific polyclonal rabbit antiserum (1:200) followed by HRP-conjugated goat anti-rabbit IgG (1:1000).
Lane 1, immune serum; Lane 2, pre-bleed serum.

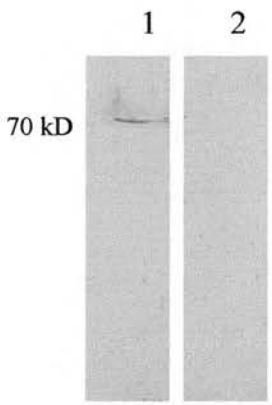


Fig. 3.10 Immuno-blot of SDS-PAGE separated schizont culture supernatant probed with mhsp70-specific polyclonal rabbit antiserum (1:200) followed by HRP-conjugated goat anti-rabbit IgG (1:1000).
Lane 1, immune serum; Lane 2, pre-bleed serum.

3.4 Discussion

The goal of the study was to derive optimal conditions for metabolic labelling of *T. lestoquardi* schizonts to allow identification of putative secreted proteins in axenic culture supernatants. Three major parameters were evaluated: incubation period, culture cell density and quantity of added radioactivity. A pronounced difference in labelling intensity was observed between cells incubated in the labelling mix for 6 hrs or 18 hrs, with 3% and 13% of total label being incorporated respectively (Table 3.1). This difference is likely to be related to utilisation of intracellular stores of unlabelled methionine/cysteine in the early stages of the culture, with effective labelling only occurring when these stores are depleted.

Inefficient uptake of ^{35}S methionine/cysteine in shorter incubation could also be associated with cell cycle and rates of protein synthesis. Depending on proliferation rate of cells, some cells may need a few hours to deplete intracellular stores of methionine/cysteine, while others may need several hours or overnight. The cell cycle of prokaryotes is simple and fast. For example, in some bacteria, the cell cycle is repeated every 30 min (Lodish et al. 1999). Most eukaryotic cells take 10- 20 hrs to double in number and some replicate at a much slower rate (Lodish et al. 1999). As mentioned in section 2.3.1, TLL double in 20 hrs in normal conditions. This may explain why a short incubation time for radio-labelling was not appropriate for TLL in this study.

To enhance the sensitivity of SDS-PAGE analysis of parasite proteins in culture supernatants, it was considered necessary to maximise the numbers of infected cells in the labelling reaction. A range of cell densities from 1×10^6 to 8×10^6 cells/ml was evaluated. The highest density consistent with adequate viability was found to be 2×10^6 cells/ml, and this also gave rise to effective metabolic labelling with 20 μCi/ml (Fig. 3.2 and 3.3). However, an increase in ^{35}S isotope uptake was observed when the amount of added radiolabel was increased from 20 μCi/ml to 40 μCi/ml. In principle, the amount of ^{35}S isotope could be increased further; no maximum amount of radioactivity is described in literature. However, it is likely that higher

concentrations of isotope are deleterious to cell viability. Since 20 μCi/ml has been used previously to label *T. parva*-infected cells (Sugimoto et al. 1989), and a significant increase in the uptake of activity was observed using 40 μCi/ml of the isotope in the present study (Fig. 3.4), this concentration was chosen for subsequent labelling experiments.

In the present study, ^{35}S incorporation in host cell and intracellular schizonts was evaluated both by scintillation counting and visualisation by SDS-PAGE and autoradiography. Scintillation counting measures the total ^{35}S methionine/cysteine taken up by cells including that stored intracellularly and that bound to cell membranes. Therefore, it does not provide an accurate indication of the extent of labelling cellular proteins, which is more readily visualised by SDS-PAGE and autoradiography. The combination of two methods ensured adequate measurement of ^{35}S incorporation by cells and cellular proteins.

Normally, cellular incorporation of radioactivity is no more than 25%, and 15-20% is regarded as efficient labelling (York I. personal communication). In the present study, 19.25% and 4.5% incorporation of TLL and schizonts, respectively, were obtained and a number of radio-labelled cellular protein bands were detected by SDS-PAGE followed by autoradiography (Fig. 3.4 and 3.5). This suggests that an adequate degree of metabolic labelling was achieved using this protocol.

A number of putative secreted schizont proteins were detected in labelled schizont culture supernatant (Fig. 3.6). These soluble proteins were only barely detectable after 6 hrs culture but were more abundant after longer incubation (18 hrs). Only a proportion of the proteins present in whole schizonts were represented in the supernatant fraction. To confirm that schizont culture supernatant contains known secreted proteins of *Theileria* schizonts, rabbit sera raised against two *T. annulata* secreted proteins, SA and SuAT, were evaluated for recognition of *T. lestoquardi* homologues in schizont culture supernatants. Both rabbit antisera recognize proteins of the appropriate size in supernatants of *T. lestoquardi* schizont cultures. This

provided evidence that cultured schizonts are capable of secreting proteins into the culture medium.

Two known secretory inhibitors, BFA and monensin, were used to determine whether radio-labelled soluble proteins were actively secreted by schizonts. Neither BFA nor monensin had an apparent inhibitory affect on the elaboration of schizont proteins into the culture supernatants (Fig. 3.8). This conflicts with the observation of known secreted proteins in the culture supernatant described above and with the absence of labelled protein in supernatants from 4 °C cultures. Although the reasons behind this observation are not clear, it may reflect an insensitivity of *Theileria* secretory pathways to these reagents.

In eukaryotic cells, there are two categories of secretory pathways, classical and non-classical ones. Classical secretion includes regulated and constitutive secretion, in which secreted proteins have signal sequences and utilise the endoplasmic reticulum (ER)/Golgi system (Rothman 1994). Non-classical secretion occurs in the absence of a functional ER/Golgi system and proteins secreted in this manner do not have signal peptides (Flieger et al. 2003). Monensin and BFA block secretion of proteins with signal sequences that utilise the classical pathway (Schuerwegh et al. 2001; Klausner et al. 1992). Little is know about the ER/Golgi system and secretory pathways in *Theileria* parasites. In the malaria parasite, a non-classical secretory pathway has been found as well as an organelle termed secondary ER of apicomplexa (sERA) that is involved in protein secretion. However, the origin of sERA, the targeting of proteins to sERA, and the fate of proteins after export to sERA have not been determined (Wiser et al. 1997).

Most of hsps in eukaryotic cells have been found to be secreted with in the absence of signal sequences and BFA and monensin inhibitors have no influence on hsp70 secretion (Multhoff G. personal communication; cited in Zugel et al. 1999). The observation of no apparent effect of BFA and monensin on schizont secretion (Fig. 3.8) implies that schizonts may utilise non-classical secretory pathways.

The failure of BFA and monensin to inhibit elaboration of labelled proteins into the culture supernatant could also reflect contamination of purified schizonts with host proteins. It is known that components of NF-κB activating complex associate with schizonts of *T. parva* within the infected cell (Heussler et al. 2002). It is likely that such proteins would copurify with schizonts. In the event that they were to dissociate from the parasite during culture, they could account for a proportion of the labelled proteins observed in culture supernatants in this study, and their presence would not be affected by secretory inhibitors.

In summary, an optimised method for metabolic labelling of TLL and intracellular schizonts has been established. Viable schizonts can be cultured in vitro for up to 18 hrs, and putative secreted proteins can be detected from schizont culture supernatant. The latter include *T. annulata* homologues, SuAT and mhsp70. Radio-labelled soluble proteins accumulate slowly in the culture supernatant and this is inhibited when schizonts are cultured at 4 °C. These results suggest that cultured schizonts are capable of secreting proteins into the culture medium. However, elaboration of protein into the supernatant is not efficiently blocked by the secretory inhibitors, BFA and monensin, suggesting that schizont secretory pathways may be non-classical. Alternatively, a proportion of labelled proteins in culture supernatant may be derived from host proteins that associate closely with the parasite in the infected cells. Further analysis of these issues is described in the following chapters.

Chapter 4: Analysis of schizont secreted proteins by mass spectrometry

4.1 Introduction

As described in the previous chapter, a method for metabolic radio-labelling TLL was established and *T. lestoquardi* schizont associated proteins labelled with the ^{35}S isotope were detected in schizont culture supernatant. The present chapter describes the analysis of proteins present in schizont culture supernatant using proteomics, which is now widely used for identification and characterisation of proteins from different organisms (Mann et al. 2001). Proteomics has been successfully used in characterising the proteomes of a number of pathogens, including *Saccharomyces cerevisiae* (Shevchenko et al. 1996), *Mycobacterium bovis* (Jungblut et al. 1999) and protozoan parasite *Toxoplasma gondii* (Cohen et al. 2002).

Two-dimensional electrophoresis (2DE) is a powerful tool of proteomics and is used for isolating proteins for further characterisation by mass spectrometry. 2DE separates proteins according to two independent properties in two separate steps: the first-dimensional step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (PI); the second-dimensional step, SDS-PAGE, separates proteins according to their molecular weights (MW). Compared to one-dimensional electrophoresis (1DE), this allows proteins to be separated from complex mixtures as single spots on the basis of their PI and mass rather than a band relating to a single mass.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) is a mass spectrometric technique widely used to generate peptide profiles of trypsin-digested proteins, in which peptides incorporated in a matrix are ionized by a laser pulse. Ionized peptides accelerate towards a detector at different rates according to their mass/charge (m/z) ratio, with smaller ions travelling faster than larger ones. The numbers of peptides detected and their calculated masses constitute a peptide mass

fingerprint (PMF), which can be used to search against theoretical trypsin-digested peptide masses of entries in protein databases (Karas 1996). As a result of its simplicity, mass accuracy, high resolution and sensitivity, MALDI-TOF is rapidly becoming the premier tool for protein identification since invented by Karas et al. in the 1980s (Egelhofer et al. 2002).

In a previous analysis of *T. parva*-infected cells using 2DE, changes in protein expression associated with parasite-induced transformation were examined (Sugimoto et al. 1988; 1991). Ten proteins were found in *T. parva*-infected cells which were absent in uninfected cells, and seven of these were detected in preparations of schizont proteins. Other minor changes in protein and glycoprotein patterns were also observed (Sugimoto et al. 1991). 2DE analysis has also been performed in other *Theileria* species, such as *T. buffeli*, *T. sergenti*, and *T. orientalis* whose schizonts and piroplasms are morphologically indistinguishable from each other. Distinct protein patterns were found for the different species (Sugimoto et al. 1991). Although these studies were performed by 2DE, they only provided protein expression patterns and no novel proteins were identified from either *Theileria* parasites or *Theileria*-infected cells.

This chapter describes the use of 2DE and MALDI-TOF MS analysis to characterise proteins elaborated by isolated schizonts during overnight culture. Because it was not possible to analyse radio-labelled material on the Moredun Research Institute (MRI) MALDI instrument, spots on Commassie-stained 2D gels prepared from whole schizonts were examined instead. Relevant spots were identified by comparison of Coomassie-stained gels with 2D autoradiographs derived from radio-labelled culture supernatants.

4.2 Materials and methods

4.2.1 Supernatant preparation

Schizonts were separated from 2×10^7 ^{35}S -labelled TLL and overnight schizont culture supernatant was collected as previously described in section 2.2.4 and 3.2.4.

4.2.2 Schizont preparation

Schizonts were separated from 2×10^7 unlabelled TLL as described in section 2.2.4, resuspended in 1 ml complete IMDM, divided between two 1.5 ml eppendorf tubes, washed with HBSS twice and pelleted at 10, 000 rpm (Microfuge, Beckman) for 3 min. As an important factor in efficient separation of individual proteins by 2DE is protein solubility, two methods of cell disruption, from gentle to vigorous lysis, were investigated to solubilise schizont proteins.

Freeze-thaw: one schizont pellet as prepared above was rapidly frozen in liquid nitrogen and thawed at RT. This procedure was repeated 3 times. The freeze-thaw treated sample was mixed with 300 µl lysis buffer (8M Urea, 4% CHAPS, 40 mM Tris base) and shaken on a MS2 Minishaker (IKA) at RT for 30 min.

Sonication: the second schizont pellet was resuspended in 300 µl lysis buffer, shaken as above for 30 min and then sonicated (Ultrawave sonicator, UK) in a waterbath at 10 °C for 5 min. Ultrosonic waves generated by the sonicator lyse cells, which result in the disruption of cells.

Lysed samples prepared by the both methods were centrifuged at 12, 000 rpm (Microfuge, Beckman) for 20 min and the supernatant was collected and concentrated as described in section 3.2.4 for 2DE analysis. The protein concentration of the sample was determined using a DU-600 Spectrophotometer (Beckman) with a 1 cm path length. An aliquot of 100 µl diluted sample (1:100 dilution in PBS) was added to a quartz cuvette and absorbance was measured at 280 and 260 nm according to the

method described by Stoscheck (1990). The protein concentration was calculated using the following formula:

Protein concentration (mg/ml) = $[(A_{280} \times 1.55) - (A_{260} \times 0.76)] \times$ dilution factor

4.2.3 Two-dimensional electrophoresis (2DE)

4.2.3.1 First-dimension of 2DE

All materials were obtained from Amersham Biosciences unless indicated otherwise. For soluble proteins present in schizont culture supernatants, samples were processed using the standard method for 2DE as described below.

Samples of radio-labelled culture supernatants (~1×10^6 cpm) prepared as described in section 3.2.4 were mixed with 75 µl rehydration buffer (8 M Urea, 2% CHAPS, 1% IPG buffer) containing a few grains of bromophenol blue per ml, and shaken on a MS2 Minishaker (IKA) for 30 min. The mixture was centrifuged at 12, 000 rpm (Microfuge, Beckman) for 2 min and the supernatant pipetted into an Immobilized pH gradient (IPG) strip holder. The IPG strips (pH3-10) were then placed onto the sample solution, with the gel side facing down and the pointed (anodic) end directed toward the pointed end of the strip holder. Finally, IPG strips were overlaid with ~1.5 ml drystrip cover fluid and the cover was placed on the strip holder.

The IPG strips were rehydrated on an IPGphor isoelectric focusing system for 12 hrs prior to protein separation. Isoelectric focusing (IEF) was performed by gradually increasing the voltage across the IPG strips to at least 3500 V and maintaining this voltage for at least 3 thousand volt-hours, with constant current of 50 µA per IPG strip. The IEF program was set up as follows:

30 V, 1hr; 150 V, 1hr; 300 V, 1hr; 600 V, 1hr; 1200 V, 1hr; 2400 V, 1hr; 8000 V, 10hrs.

4.2.3.2 Second-dimension of 2DE

After IEF, the IPG strips were soaked in equilibration buffer (50 mM Tris-Cl, pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, 10 mg/ml of dithiothreitol) containing a few grains

of bromophenol blue per ml for 15 min. The reductant, dithiothreitol (DTT), ensures that disulfide bridges are broken (Gorg et al. 1988). The buffer was decanted and a second equilibration was performed in 15 ml equilibration buffer containing iodoacetamide (20 mg/ml) for another 15 min to prevent protein reoxidation during electrophoresis and reduce streaking in the second-dimension separation (Gorg et al. 1988). The strips were then rinsed with distilled water and placed on filter paper for 3 min.

With the temperature on a MultiTemp III thermostatic circulator set to 15 °C, about 3 ml IPG Cover Fluid was pipetted onto the cooling plate of Multiphor II Electrophoresis Unit, and an SDS ExcelGel (250 × 110 × 0.5 mm) was placed on the plate. The cathodic and anodic buffer strips were placed at either end of the SDS gel. The IPG strip (gel side down) was placed parallel to the cathodic strip and standard protein markers (10 µl, Biorad) were loaded adjacent to the IPG strip. Electrophoresis was performed in the following phases: Step 1. 600 volts, 20 mA, 40 W, 15 min; Step 2. 600 volts, 40 mA, 40 W, 90 min.

Different IPG strip specifications were evaluated to obtain optimal separation of schizont-derived proteins from culture supernatant. Strips of 7 cm length with linear gradients were first tried, followed by nonlinear IPG strips of 7 cm and, finally, 18 cm nonlinear strips. Once optimal 2DE conditions for schizont culture supernatant had been established, samples of schizont lysates were examined using the same method, with 40 µg of lysate being added per 7 cm IPG strip and 100 µg of lysate per 18 cm strip.

4.2.4 Visualization of proteins on 2D gels

2D gels were fixed in 200 ml 10% glacial acetic acid for 30 min by shaking on a platform shaker (Stuart Scientific Ltd, UK) before staining with Coomassie blue staining solution (1% Coomassie blue stain R250 in dH_2O/50% methanol/10% glacial acetic acid) for 1 hr. Gels were then destained in dH_2O/25% methanol/10% glacial acetic acid for at least 1 hr.

2D gels containing radio-labelled proteins derived from schizont culture supernatants were removed from the plastic supporter and dried onto filter paper under vacuum at 80 °C (Hoefer SLAB GD2000) for 1 hr. The dried gel was exposed for 5 days to FUJI film (FUJI Ltd) in a cassette at -20 °C, after which the film was developed in an automatic X-Ograph Compact X2 (X-Ograph Ltd, UK). Radio-labelled proteins were visualised and compared with spots in Coomassie stained gels of schizont lysates.

4.2.5 Identification of corresponding spots on Coomassie stained gels

When comparing spots on autoradiographs of ^{35}S-labelled proteins with those of Coomassie stained 2D gels of schizont lysates, it was noticed that shrinkage of the gels during the drying process made it difficult to align individual spots with their counterparts on the autoradiographs.

To address this problem, 75 µl of schizont lysate was spiked with an equal volume of ^{35}S-labelled schizont culture proteins and separated by 2DE as described above. The gel was then stained with Coomassie blue, dried and exposed to X-ray film. The resulting autoradiograph was then aligned with the Coomassie-stained gel to allow accurate identification of the positions of labelled spots within the proteome.

4.2.6 MALDI-TOF analysis of putative secreted proteins

4.2.6.1 Spot processing for MALDI-TOF

Spots corresponding to those identified on autoradiograph were excised from Coomassie stained 2D gels, cut into small pieces (approximately 0.5-1 mm in diameter) and placed into clean 0.5 ml siliconised Eppendorf tubes. The pieces were then covered with 15-20 µl of 100 mM ammonium bicarbonate/50% acetonitrile and left for 15 min at RT. The supernatant was then removed and replaced with fresh 100mM ammonium bicarbonate/50% acetonitrile. This step was repeated at least 3 times until the stain had disappeared. The gel pieces were then dehydrated by soaking in 100% acetonitrile for 10 min. The acetonitrile was removed and the gel pieces were centrifuged under vacuum (Speed Vac Concentrator, Savant Instruments, US) for 20 min. The pellet was

finally mixed with 5- 10 µl trypsin (10 ng/µl in 25 mM ammonium bicarbonate) and incubated at 37 °C for overnight prior to analysis by MALDI-TOF.

4.2.6.2 MALDI-TOF analysis

MALDI-TOF analysis was performed using a MALDI-TOF Voyage DE-PRO Micromass spectrometer (Applied Biosystems, USA) equipped with a 337 nm nitrogen laser. Aliquots (0.5 µl) of the trypsin digested solution were fixed in a UV-light absorbing matrix, α-cyan-4-hydroxycinnamic acid (CHCA) onto a target plate that was loaded in the mass spectrometer. The digested sample was then ionised by laser bombardment, the TOF was recorded, and the mass/charge ratio of individual fragments was calculated. In this way, peptide mass profiles were collected for each trypsin-digested spot and used for database searches.

4.2.6.3 Database search

Database searching with the collected peptide masses was undertaken using the Mascot programme, through which peptide masses generated by theoretical trypsin digestion of database proteins are compared with those observed by MALDI-TOF. Peptide modifications allowed during the search were cysteines modified by carbamidomethylation and oxidised methionines. The maximum number of missed cleavages was set to 1. The tolerance was constrained to a peptide mass of 50 ppm in the first instance and increased to 100-200 ppm if no significant homology matches were identified.

4.3 Results

4.3.1 2DE analysis of ^{35}S-labelled soluble proteins present in the culture supernatant

^{35}S-labelled proteins present in schizont culture supernatant were processed using the standard methodology for 2DE analysis. In initial experiments, 7 cm linear IPG strips with a pH range of 3-10 were used, as the majority of proteins would have their isoelectric points in this wide range.

Under these conditions, approximately 10 spots were observed in autoradiographs of 2D. However, some spots were clustered and difficult to resolve (Fig. 4.1 A). Nonlinear IPG strips, with the same pH range of 3-10, were then evaluated because nonlinear strips improve resolution between pH 5 and pH 7. Nonlinear IPG strips were observed to provide better resolution, with more spots being separated than with linear strips (Fig. 4.1 B).

Nonlinear IPG strips (pH 3-10) with longer sizes (18 cm) were then evaluated to improve the resolution further and it was observed that more spots were resolved under these conditions (Fig. 4.2). In total, around 35 spots were detected by 2DE autoradiography of schizont culture supernatants, with the most dominant spots being between 60 kDa and 80 kDa in mass, with PI ranging between pH 4.0 and pH 8.0 (Fig. 4.2). However, protein species were present in small amounts, requiring 5 days exposure for visualisation.

4.3.2 2DE analysis of *T. lestoquardi* schizont lysates

In view of the small quantities of schizont-derived proteins present in culture supernatants and the fact that it was not possible to load radio-labelled material on to the MRI MALDI-TOF instrument, spots corresponding to those present on autoradiographs were identified in 2D gels of whole schizont lysates and processed for MALDI analysis.

Effective separation of proteins by 2DE requires efficient solubilisation and two different methods, freeze-thaw and sonication, were evaluated for accomplishing this. It

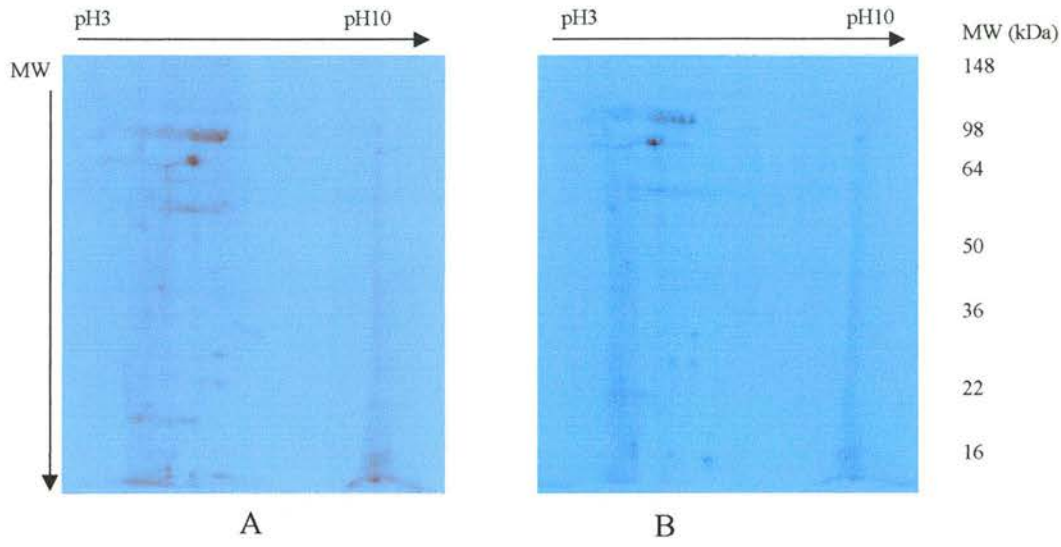


Fig. 4.1 2D autoradiographs of ^{35}S- labelled proteins from purified *T. lestoquardi* schizont culture supernatants

A. IPG strip pH3-10, 7 cm, linear; B. IPG strip pH3-10, 7 cm, nonlinear. Each gel was loaded with 1×10^6 cpm sample.

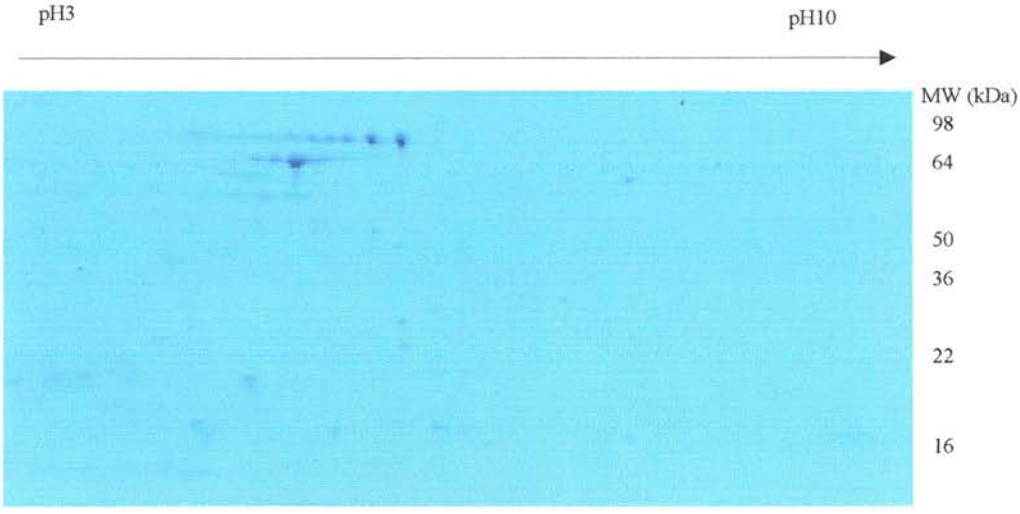


Fig. 4.2 2D autoradiograph of ^{35}S - labelled proteins from purified *T. lestoquardi* schizont culture supernatant (2×10^6 cpm)

IPG strip pH3-10, 18 cm, nonlinear.

was observed that the freeze-thaw method was not adequate for solubilisation. Schizont proteins appeared to aggregate under these conditions and failed to separate completely during 2DE (Fig. 4.3 A). The sonication disruption method was found to be superior for solubilising schizont proteins and schizont lysates treated in this way were observed to separate efficiently in 2D gels. This is illustrated in Figure 4.3 B, which shows a 2D gel prepared from unconcentrated material and hence contains a limited number of visible spots.

More spots were observed from the concentrated samples separated by 2DE (Fig. 4.4). This method was thereafter used routinely for schizont lysis and sample preparation for 2DE in the study. The 2DE conditions used for schizont lysates were similar to those used to separate ^{35}S-labelled schizont culture supernatants. IPG strips with a wide pH range (pH 3-10) and 7cm or 18 cm in length were used.

Examination of 2D maps of schizont lysates revealed that proteins were resolved and separated more efficiently using 7 cm IPG strips with nonlinear gradients from pH3-10 than those with linear gradients, with 50 and 45 spots being observed respectively (Fig. 4.4 A and B). Using 18cm nonlinear strips (pH 3-10), approximately 60 spots could be detected in Coomassie blue stained 2D gels, indicating that more spots were resolved than with 7 cm strips (Fig. 4.4 C). The most abundant spots were observed in the region of 60-80 kDa, between pH 4.0 and pH 6.0. From these results, 18 cm nonlinear strips (pH3-10) were found to be most effective for resolving schizont proteins and were used for separation of schizont lysates in subsequent experiments.

4.3.3 Identification and confirmation of corresponding spots in Coomassie stained gels

2D autoradiographs of ^{35}S-labelled proteins from schizont culture supernatants were aligned with 2-D maps of Coomassie stained schizont lysates and approximately 20 common proteins were identified based on the analysis of spot patterns. The dominant spots appeared in the same area in both samples (Fig. 4.5). Approximately 10 spots were observed in the schizont culture supernatant preparations that were not present in the schizont lysate preparation.

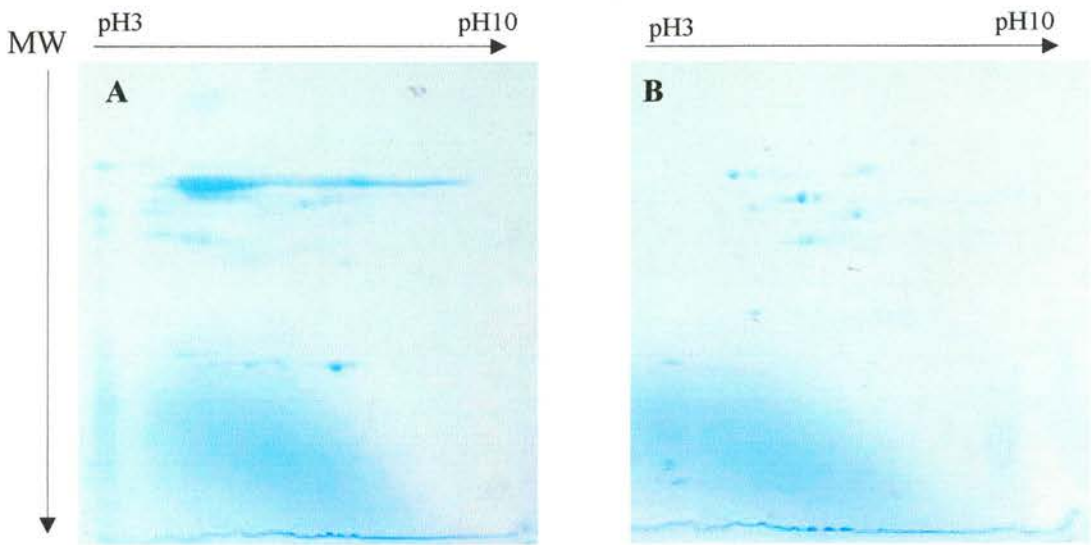


Fig. 4.3 2D maps of purified *T. lestoquardi* schizont lysates using freeze-thaw (A) and sonication lysis (B)

IPG strip pH3-10, 7 cm, linear.

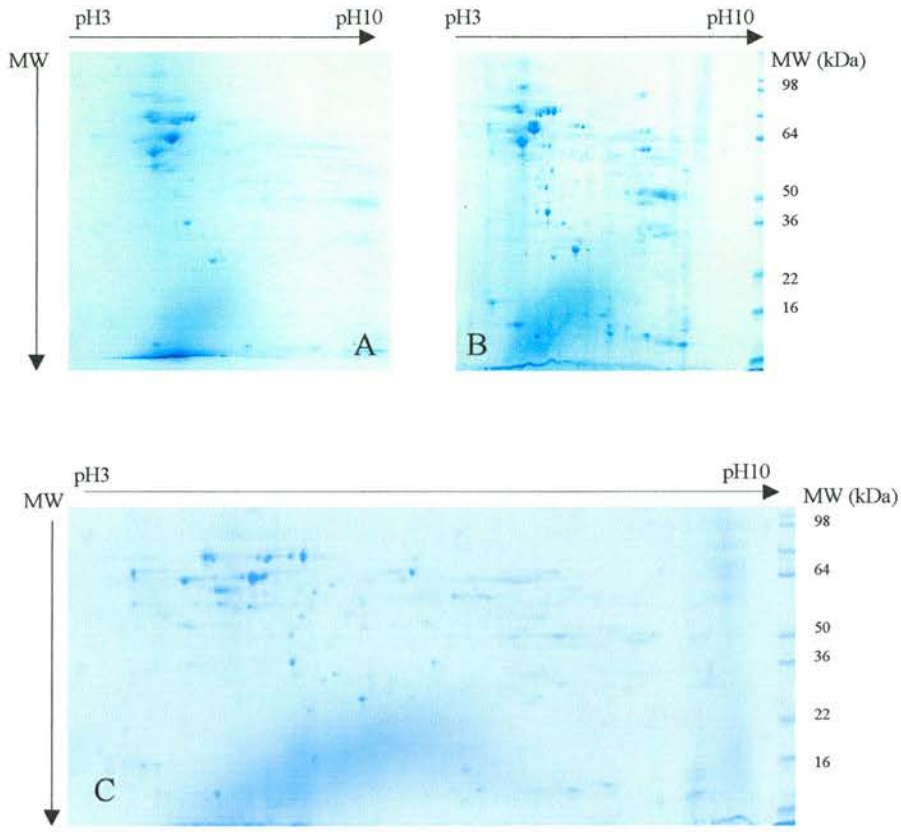


Fig. 4.4 2D maps of purified *T. lestoquardi* schizont lysates

A. IPG strip pH3-10, 7 cm, linear; B. IPG strip, 7 cm, nonlinear; C. IPG strip 18 cm, nonlinear. Each 7cm strip was loaded with 40 μg sample and 18cm strip was loaded with 100 μg sample.

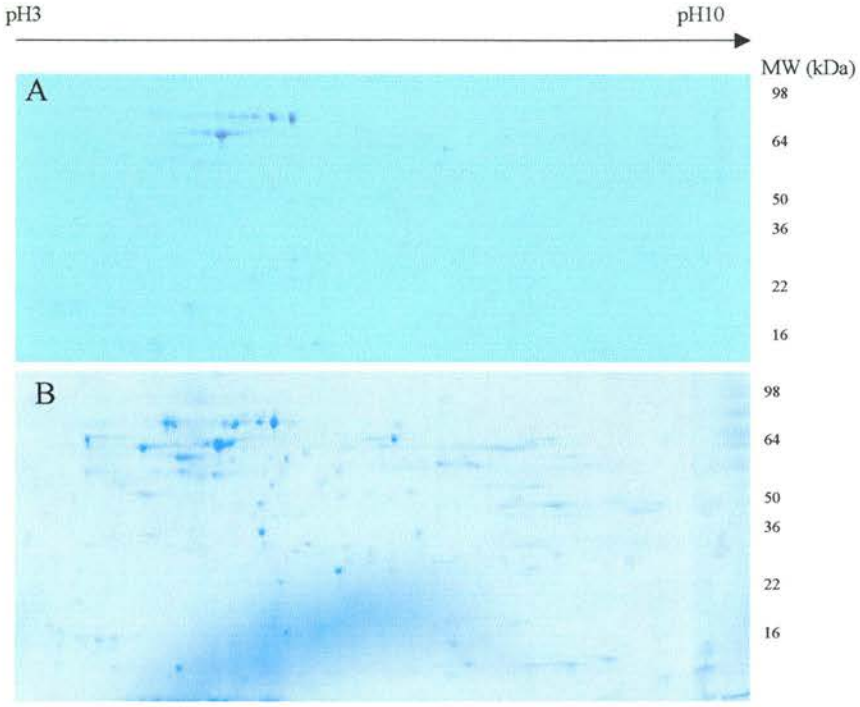


Fig. 4.5 2D map alignmentof ^{35}S-labelled supernatant proteins with Coomassie stained schizont lysates

The 2D autoradiography of ^{35}S - labelled proteins from schizont culture supernatant (2×10^6 cpm, A) was aligned with Commassie stained gel of *T. lestoquardi* schizont lysates (100 μg, B).

However, some shrinking and distortion occurred during the drying of 2D gels so that spots from autoradiographs were difficult to align with the Coomassie stained gel. Because this shrinkage made it impossible to align autoradiographs and gels accurately, a spiking approach was adopted to confirm the identity of common spots.

More spots were observed in gels prepared from the spiked mixture than had been seen in previous gels (Fig. 4.6). This was expected given that complete IMDM medium also contains FCS proteins. Comparison of the dried Coomassie-stained 2D gel prepared using schizont lysate spiked with ^{35}S-labelled schizont culture supernatant proteins with the autoradiograph derived from it allowed accurate identification of 16 common spots of adequate staining intensity for MS analysis. These common spots were designated TLS1-16 (Fig. 4.7).

4.3.4 MALDI-TOF MS analysis

Spots identified by 2DE from schizont lysates were trypsin-digested and analysed by MALDI-TOF. Using the Mascot software, peptide mass profiles from individual spots were compared with calculated peptide masses of each record in available databases. Profiles were searched against the general Swiss-prot database (http://ca.expasy.org/sprot/) and matched homologues were subsequently blast-searched against the *T. annulata* genome database (http://www.sanger.ac.uk/Projects/T_annulata/).

The accuracy of homology hits were evaluated in each case on the basis of the Mowse score value and the predicted molecular weight (MW) and isoelectric point (pI) of the putative homologue as compared with the position of the original spot on the 2D gel. Candidate proteins identified for eight of the common spots are listed in Table 4.1, along with corresponding Mowse scores. Each Mascot search result provides a value above which all searches are significant at the $p<0.05$ level, so that, if the score for a particular match exceeds the significance level, there is less than a 1/20 chance that the searched match is a random event.

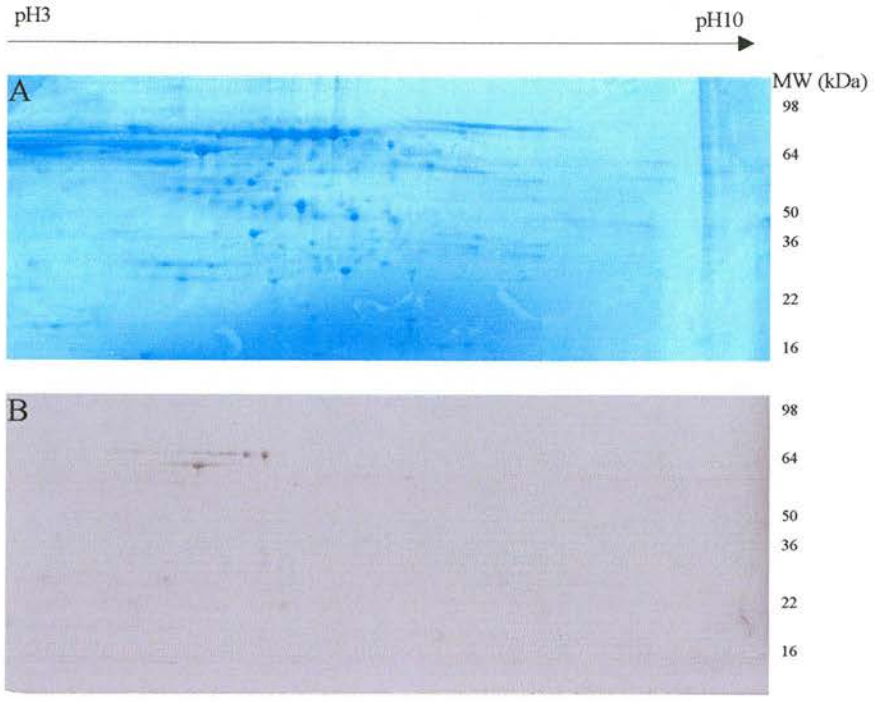


Fig. 4.6 Confirmation of identified corresponding spots using the spiking method

A. The 2D map of purified *T. lestoquardi* schizont lysates (100 μg) mixed with ^{35}S labelled schizont culture supernatant (2×10^6 cpm).

B. 2D autoradiograph derived from A.

IPG strip pH 3-10, 18 cm, nonlinear.

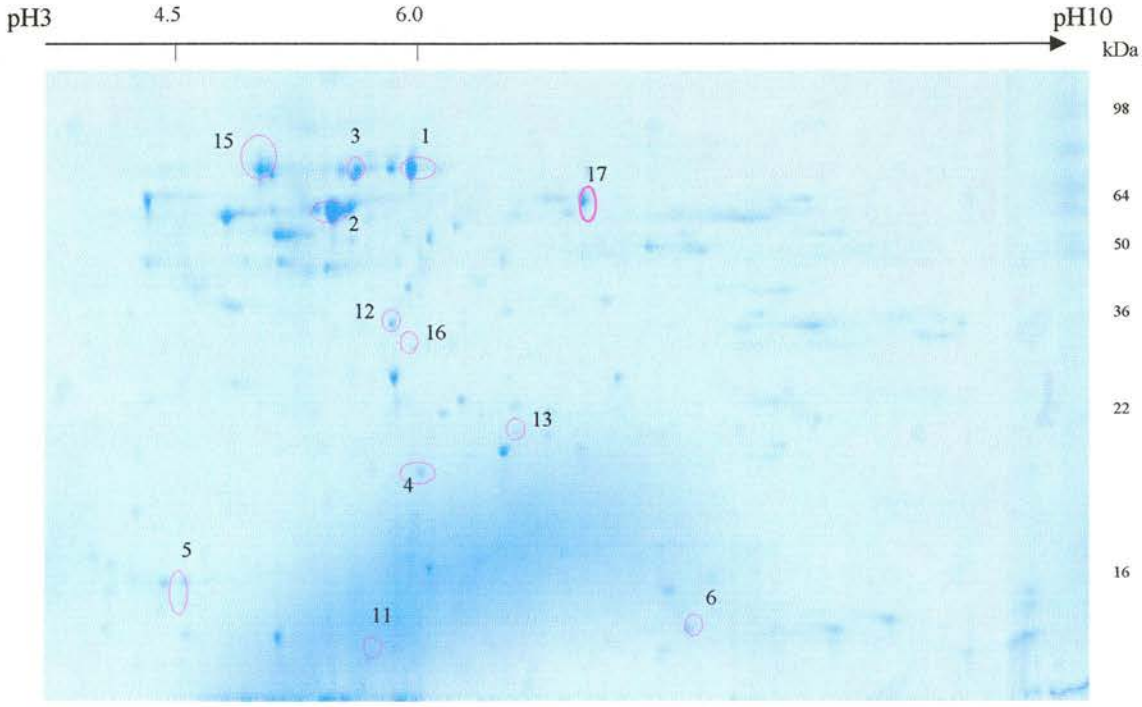


Fig. 4.7 2D map of *T. lestoquardi* schizont lysates stained by Coomassie blue
Spots in common with those of autoradiographs derived from radio-labelled schizont culture supernatant are circled and numbered as described in the text. Specific pH points are indicated, based on pI of known proteins in the map.

Spot No.	Mowse score	Significance threshold (P<0.05)	Identified protein homologues	Protein accession No.
TLS1	101	63	Stress-70 protein, mitochondrial precursor, human	P38646
TLS2	103	63	60 kDa heat shock protein, mitochondrial precursor, human.	P10809
TLS3	53	63	mitochondrial heat shock protein hsp70, *Theileria.*	AAQ13186
TLS4	62	63	60S ribosomal protein L12, mitochondria, bovine.	NP081480
TLS5	33	63	ATP synthase delta chain, mitochondrial precursor, bovine	P05630
TLS11	40	63	ATP synthase coupling factor 6, mitochondria, bovine	P13618
TLS12	53	63	pyruvate dehydrogenase (lipoamide) beta, porcine	1917268B
TLS15	121	63	Heat shock 70 kDa protein, *Theileria.*	P16019

Table 4.1 *T. lestoquardi* associated proteins identified by MALDI-TOF analysis

4.4 Discussion

In this study, 2DE was employed to separate *T. lestoquardi* derived proteins present in supernatants of overnight schizont cultures and to purify them from schizont lysates for MS analysis. By comparing 2D autoradiographs of ^{35}S-labelled proteins from overnight schizont cultures with 2D gels of total schizont lysates, 16 common spots were identified for excision from the gels and subsequent MALDI-TOF analysis.

Proteins secreted by *Theileria* parasites are potentially involved in signalling pathways for host cell transformation and are also candidate antigens for CTL recognition (Nene et al. 2000). The present study aimed to identify parasite proteins in schizont culture supernatant on the basis that these are likely to be secreted, and to determine their identity by MS analysis. However, in vitro culture of schizonts required complete IMDM (section 2.3) in which FCS (5-10 %) and other amino acids were present and it was anticipated that secreted schizont proteins would be present in much smaller amounts. It was therefore decided to metabolically label the schizonts with ^{35}S prior to culture to increase sensitivity and to allow discrimination of parasite proteins from those present in the media.

MS was employed in the present study for analysis of identified spots. As radio-labelled spots visible in autoradiographs are below the detection threshold of the MS analysis, it was necessary to identify these spots in 2D gels of whole schizonts, which can be prepared with much larger amounts of protein. Around 60 spots were detected in gels prepared from unlabelled schizont lysates stained with Coomassie blue (Fig. 4.7). It is obvious that these spots represent dominant schizont proteins rather than whole schizont proteome. The small number of spots (~60) detected in schizont lysates in this study clearly limited the numbers of spots available for analysis.

Several reports have described identification of larger number of spots by 2DE and MS. Over 1000 spots were resolved from *T. gondii* tachyzoite on a silver stained 2D gel (Cohen et al. 2002). From *M. tuberculosis*, 1800 and 800 proteins were observed

respectively from Mycobacterial cell proteins and culture supernatant by 2DE analysis (Jungblut et al. 1999). 200 individual spots were observed in a Coomassie blue stained 2D gel prepared from excreted/secreted proteins of *Haemonchus contortus,* a nematode that infects small ruminants. With silver staining, more spots (950 spots) were detected. A total of 107 proteins were subsequently identified from the 200 Coomassie-stained spots using different MS methods (Yatsuda et al. 2003).

There are a number of possible reasons for the relatively small number of schizont spots identified in *T. lestoquardi* schizont lysates. Firstly, although 2DE offers greater protein resolution potential than any other current separation technique (Gorg et al. 2000), it still has disadvantages. Due to phenomena not completely understood, membrane and hydrophobic proteins can be poorly represented in the 2nd dimension of 2DE (Adessi et al. 1997), which probably results from protein/gel interactions during IEF. Secondly, extreme proteins (larger than 150 kDa and smaller than 10 kDa) are poorly resolved. Some larger proteins may also be lost and it has been suggested that this is due to size exclusion when the proteins are loaded onto the second-dimension gel (Simpson 2003). Thirdly, the 2D gels in the present study were stained by Coomassie blue, which is relatively insensitive compared to silver staining. Coomassie stains mainly abundant proteins and it is estimated that its staining threshold is around 0.2-0.5 μg (Wilson 1983). Silver staining can provide higher sensitivity (50-fold greater sensitivity than Coomassie staining) so that protein spots of about 2-10 ng can be detected (Rabilloud et al. 1994). This approach would clearly be preferable to obtain a 2D map profile from whole schizont proteins. However, sliver staining is not compatible with MS analysis (Rabilloud et al. 1994) and hence was not appropriate for protein identification in this study. Another possible reason for the small number of *T. lestoquardi* schizont proteins detected by 2DE may be the quantity of schizont proteins applied to the first dimension. The balance between yield and purity of schizonts was an important consideration for the present study, and the requirement for high purity of separated schizonts constrained the availability of large amount of schizont protein material.

In a previous study, protein expression was compared in *T. parva*-infected cells and uninfected cells using metabolic labelling and 2DE. In total, only 10 proteins were found in infected cells that were not present in un-infected cells (Sugimoto et al. 1989), which implies low amounts of expressed schizont proteins. None of these differentially expressed proteins were identified thereafter. Although schizonts were purified from infected cells and schizont proteins were also analysed by 2DE method, no description is provided of how many spots were obtained from schizont preparations (Sugimoto et al. 1989).

As described above, around 60 spots were detected in 2D gels of purified schizonts, which is a small number compared to theoretical complement of schizont expressed proteins (1169 are estimated from the annotated *T.annulata* genome, Hall N. personal communication). However, as the main aim of this study was to identify dominant schizont secreted proteins and the spots detected in 2D gels of *T. lestoquardi* schizonts represented the most dominant proteins, these spots were considered to satisfy the requirements of the project.

Schizont lysate proteins corresponding to those present in schizont culture supernatants were digested with trypsin and analysed by MALDI-TOF. MALDI-TOF analysis is widely used to identify proteins through a technique known as peptide mapping (Karas 1996). This method is based on matching observed peptide masses with those of calculated trypsin peptide masses of each entry in a given sequence database. In total, 16 putatively secreted *T. lestoquardi* proteins were analysed by MALDI-TOF. Of these, 8 gave homology matches (Table 4.1), while others gave weak or no matches (data not shown). Database searches were therefore unfruitful for 50% of the spots analysed by MS. There are several possible reasons for this. The first relates to the small quantities of proteins available in some of the spots. Stronger signals are obtained with the more abundant proteins and give a greater chance of positive identification. As mentioned above, the requirement for schizont purity constrained parasite yields and hence quantities of available protein. Other possible reasons include incomplete trypsin digestion, or contamination of the trypsin digest with other proteins, leading to complex peptide profiles and consequent unproductive database searches.

Unfruitful database searches could also have resulted from database limitations. It is possible that some of the proteins had not previously been characterised and were therefore absent from existing databases. As more genomes from different organism are analysed, databases of proteins and genes are expanding so that currently unknown proteins could be identified in the future. Although the *T. annulata* genome sequence was accessible at the time of these experiments (http://www.sanger.ac.uk/Projects/T_annulata/), it had not been annotated. The Swiss-prot database was therefore used in the first instance and homologous proteins were thereafter blast-searched in the *T. annulata* genome. Among the 8 proteins for which matches were obtained, only two were of parasite origin, *Theileria* mhsp70 (TLS3), *Theileria* hsp70 (TLS15); both were available in the Swiss-prot database. The others were all of mammalian and presumably host origin (Table 4.1). As the *T. annulata* genome has now been annotated (http://www.genedb.org/genedb/annulata/index.jsp), this provides much better opportunities for *Theileria* protein identification.

Another possible reason for the unproductive database searches is limitations associated with the MALDI-TOF technology. MALDI-TOF has been used widely for identification unknown proteins because of its simplicity, high resolution and sensitivity (Egelhofer et al. 2002). However, it relies on peptide fingerprinting, with database searches based on probability scoring. Proteins are identified by matching a list of experimental peptide masses with the calculated peptide masses for all entries in the sequence database. Peptide mass fingerprinting can only provide the most statistically probable matches and not a definitive identification. Of the 8 matched homologues identified in this study, 5 had only low scores and were not significant matches (Table 4.1). Therefore, these proteins required alternative methods to confirm their identities. This could be achieved, for example, by direct N-terminal sequencing or through Quadrupole-time of flight (Q-TOF) analysis to determine the sequence information of the individual peptides contained in the digest mixture, with which further database searching could be carried out and protein identities obtained. Application of these methodologies to identification of unmatched spot proteins is described in the following chapter.

Chapter 5: N-terminal sequencing and Q-TOF analysis of schizont associated proteins

5.1 Introduction

Establishment of methods for purification and metabolic labelling of *T. lestoquardi* schizonts described in previous chapters allowed the use of 2DE to identify 16 protein species putatively secreted by *T. lestoquardi* schizonts. Eight of these were allocated provisional identities as parasite associated proteins by MALDI-TOF MS analysis and database searches (table 4.1). Because MALDI-TOF MS analysis is based on peptide mass fingerprinting (PMF) of trypsin-digested protein, it provides only an indicative identity based on statistical probability. It is therefore difficult to identify highly conserved proteins using this method. Among the parasite associated proteins identified by the MALDI-TOF analysis, the most abundant were TLS1, TLS2, TLS3 and TLS15, with respective homologies to human heat shock protein (hsp) 70, human hsp60, *Theileria* mhsp70 and *Theileria* hsp70. As hsps are highly conserved between species, trypsin digested PMFs of each protein obtained from MALDI-TOF analysis are not sufficiently discriminatory to distinguish the *Theileria* parasite hsps from those derived from host cells.

Since its development in the 1950s, Edman degradation has become the most widely used method of direct peptide sequence analysis (Edman et al. 1967). The method is based on reaction of the polypeptide N-terminus with phenylisothiocyanate (PITC) followed by acid hydrolysis of the N-terminal amino acid from the polypeptide, yielding a cyclic phenylthiohydantoin (PTH) derivative and the polypeptide, which is shorter at its N-terminus by one residue. Because the reaction does not destroy the remaining peptide chain, a sequential degradation of the peptide can be performed. Each amino acid PTH derivative is then identified by high-performance liquid chromatography (HPLC) (Edman et al. 1967). As the degradation starts from the protein N-terminus, it is also called N-terminal Edman degradation sequencing or N-terminal sequencing.

Usually, 4-5 amino acid residues obtained from Edman degradation are sufficient to search protein databases and confirm the identity of the protein in question. This method was therefore considered a strong option for confirming the identity of hsps identified by MALDI-TOF in the present study.

Although Edman degradation sequencing has its advantages and is the classical method used to identify proteins, it still has limitations. In practice, many polypeptide chains have blocked amino termini and are hence refractory to the Edman degradation procedure. It has been estimated that greater than 75% of intracellular proteins isolated from eukaryotic cells have blocked amino termini, mainly as a result of post-translational modification (Brown 1979). Secondly, the minimum amount of protein required for N-terminal sequencing is 10-50 picomoles and, in some instances, more than 100 picomoles are necessary (Simpson 2003). This constrains the identification of proteins that are difficult to obtain in sufficient quantity. Therefore, it is preferable to use a more sensitive method capable of providing internal sequence information. This can be achieved by Tandem mass spectrometry (also called MS/MS), in which peptides resulting from digestion with a chosen enzyme can be further fragmented into ions (Peng et al. 2001). This strategy offers the possibility of obtaining multiple peptide sequence information from various regions of the polypeptide chains.

Quadrupole-time of flight (Q-TOF) is a powerful form of Tandem MS, the principle advantage of which is that a family of multiple charge states of the ion can arise from a single precursor. These multiple charged states can be used to provide more sensitive measurement than the single charged ions formed by MALDI. Because each polypeptide can be fragmented into ions in Q-TOF analysis, the information from fragment ions can then be pieced together to generate peptide sequence and structural information (Morris et al. 1997). Peptide fragmentation and sequence information can therefore provide a more accurate analysis than MALDI-TOF for identification of proteins. Q-TOF has much higher sensitivity (normally only 0.1-5 picomoles are required) than the N-terminal sequencing method and also has the capability to analyse multicomponent samples. The simplicity of the instrumentation

required for Q-TOF and its ease of operation have led to rapid acceptance of the method and to its broad application in molecular biology (Moormann et al. 1997).

As discussed in the previous chapter, no significant matches were obtained from 2DE spots of TLS4, TLS5, TLS11, TLS12, TLS13 and TLS16 by searching databases using PMF obtained from MALDI-TOF MS analysis. This chapter describes further analysis of these spots using Q-TOF MS.

5.2 Materials and methods

5.2.1 Protein transfer by semi-dry electroblotting

To prepare proteins for N-terminal sequencing analysis, 2D gels of *T. lestoquardi* schizont lysates were transferred to polyvinylidene difluoride (PVDF) membranes and stained. Schizont proteins (100 μg) were prepared as described in section 4.2.2 and separated by 2DE using 18 cm nonlinear IPG strips as described in section 4.2.3.

After the 2nd dimensional electrophoresis, the gel was removed from its plastic supporter and immersed in dH_2O. The gel was then equilibrated with anode buffer 2 (25mM Tris, pH8.3) for 3-5 min. Transfer was accomplished using a semi-dry MilliBlot-Graphite Electroblotter I (Millipore). Sheets of Whatman 3 MM filter paper were cut to fit the gel size (250 × 110 × 0.5 mm). A sheet of filter paper was soaked in anode buffer 1 (0.3 M Tris, pH 8.3) and placed on the anode plate, followed by a second sheet of filter paper soaked in anode buffer 2 and an Immobilon PVDF membrane (Westran, BioRad), which had been equilibrated in methanol for 3-5 min, rinsed with dH_2O and soaked in anode buffer 2 for 3-5 min. The gel was then placed on the membrane and covered by a sheet of filter paper soaked in cathode buffer (25 mM Tris, pH 9.4). Air bubbles were cleared from sandwich by rolling a glass pipette over the papers and gel. Electro-blotting was performed at 300 mA for 1hr.

After transfer, the PVDF membrane was rinsed in dH_2O and stained with Coomassie blue (0.5% Coomassie blue G-250 in 50% methanol/10% acetic acid) for 5 min. The membrane was then destained with dH_2O/60% methanol for 5 min, rinsed in dH_2O and dried. Spots corresponding to TLS1, TLS2 and TLS3 were identified on the basis of relative molecular mass (MW) and isoelectric point (pI) and excised from the blotted membrane using a clean scalpel and stored at -20 °C prior to N-terminal sequencing.

5.2.2 N-terminal sequencing analysis

Schizont spots were subjected to N-terminal sequencing as described by Hayes et al. (1989) and conducted by Dr Andrew Cronshaw, Edinburgh Protein Interaction

Center (EPIC), the University of Edinburgh. Sequencing was performed on an Applied Biosystems Procise 4HT instrument (Applied Biosystems, UK) with a 120A in-line PTH analyser. After Edman degradation, the PTH derivatives cleaved from the peptide were separated on an Applied Biosystems PTH C18 column run with a 0-100% (v/v) gradient of acetonitrile in 0.5% formic acid. Chromatography was performed at 55 °C and the column elute was monitored at 269 nm (Hayes et al. 1989).

5.2.3 Q-TOF analysis

2DE of schizont lysates was performed and spots prepared and digested with trypsin as described in section 4.2.6. Trypsin digested samples were analysed by Q-TOF in the Proteomics Unit of the University of Glasgow by Dr Andy Pitt. TLS4, TLS5, TLS11, TLS12, TLS13, TLS15 and TLS16 were selected for analysis.

A 10 µl aliquot of each sample digested by trypsin was introduced to a QStar Pulsar electrospray mass spectrometer fitted with a nanospray source (Protana) using a standard methodology described by Morris et al. (1997). Peptides were trapped on the C18 trap and desalted for 5 min before being separated using a 5-40% acetonitrile/0.5% formic acid gradient over 15 min. Mass spectrometric analysis was performed in Information Dependent Acquisition (IDA) mode (AnalystQS software, Applied Biosystems), and the 4 most intense ions selected for MS/MS analysis using the standard energy settings of collision-induced dissociation (CID). Masses of fragment ions were then added to the exclusion list for 3 min. Peaks were extracted using the Mascot script (BioAnalyst, Applied Biosystems) and automatically exported to the Mascot search engine.

The raw data of fragment ions from each sample were searched against the Swiss-prot databases (http://ca.expasy.org/sprot/sprot-top.html) and *T. annulata* parasite database (http://www.genedb.org/genedb/annulata/index.jsp) using the MASCOT engine, with a peptide tolerance of 1.0 kDa and an MS/MS ion tolerance of 0.5 kDa, allowing for 1 missed cleavage and variable methionine oxidation.

5.3 Results

5.3.1 N-terminal sequencing

T. lestoquardi schizont proteins were separated by 2DE and transferred to PVDF membrane by semi-dry blotting. Spots corresponding to TLS1, TLS2 and TLS3 (Fig. 5.1) were excised from the membrane and processed for N-terminal sequencing.

N-terminal sequences obtained from TLS1, TLS2 and TLS3 are outlined in table 5.1. Ten amino acid residues were obtained from spots of TLS2 and these showed strongest homology with human hsp60 in the Swiss-prot database. This suggested that TLS2 is of host origin, although sequence information on ovine hsp60 was not found in database searches. However, a bovine hsp60 fragment sequence of AKDVKFGADARALMLQGVDLLA was found that is identical to the human homologue. This provided strong evidence that TLS2 is ovine hsp60. The amino acid sequences of human hsp60 and its *T. annulata* homologue are shown in Figure 5.2.

Eight amino acid residues were obtained from TLS1 and these were identified as human mhsp70 in a search of the Swiss-prot database. As neither bovine nor ovine mhsp70 sequences are available and, since human mhsp70 has a high level of identity to those of other mammalian species, it was concluded that TLS1 is ovine mhsp70. The signal from TLS3 N-terminal sequencing was relatively poor, with only 5 amino residues being obtained. The search of the *T. annulata* database revealed that the sequence of AKVQG is identical to that of the *T. annulata* mhsp70 (Tamhsp70). Given the close relatedness of *T. lestoquardi* and *T. annulata* (Hooshmand-Rad et al. 1973; Leemans et al. 1999), it was concluded that TLS3 is the mhsp70 of *T. lestoquardi*. The sequences of TLS1 and TLS3 and their homology alignments are shown in Figure 5.3.

5.3.2 Q-TOF analysis

Fragmented ion masses from the Q-TOF analysis of each protein spot were used to search the Swiss-prot and *T. annulata* databases using the Mascot search engine. Identified proteins are listed in Table 5.2. In Mascot, the score for an MS/MS match is based on the absolute probability (P) that the obtained match between the experimental

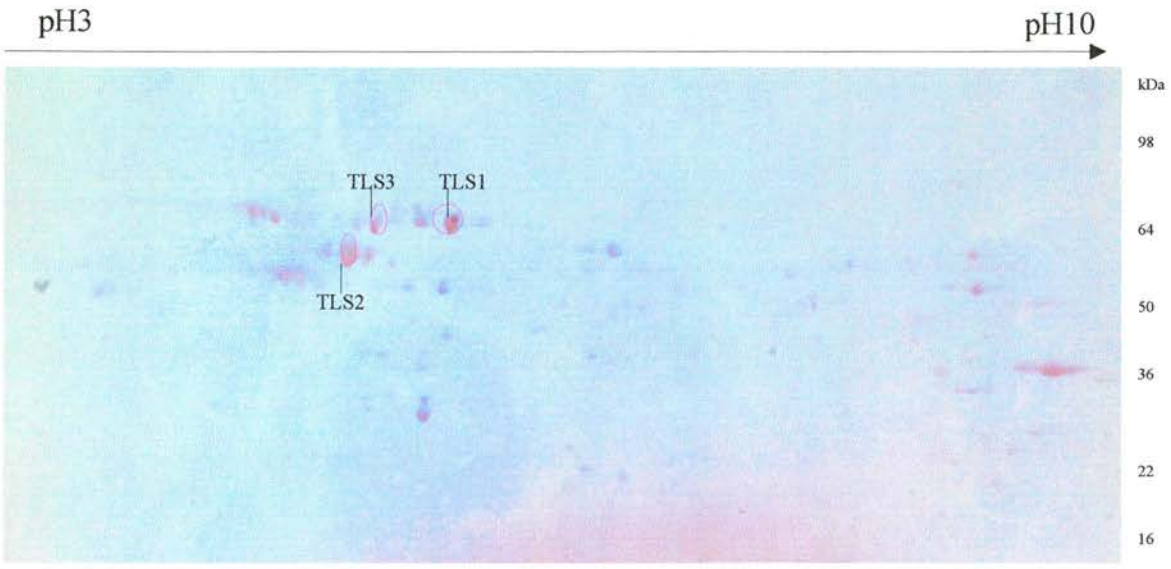


Fig. 5.1 Semi-dry blotting of a *T. lestoquardi* schizont lysate of the 2D gel

Spots of TLS1, TLS2 and TLS3 identified by MALDI-TOF analysis as homologous to human mhsp70, human hsp60 and *Theileria* mhsp70, respectively, which were selected for further analysis with N-terminal sequencing.

	Cycles									
Spots	1	2	3	4	5	6	7	8	9	10
TLS1	A/G	S	E	A	I/F	K	G	A/V		
TLS2	A/S	K/L	D	V	K/E	F	G	A	D	A
TLS3	A	K	V	Q	G/A					

Table 5.1 Amino acid residues of *T. lestoquardi* schizont proteins, TLS1, TLS2 and TLS3, identified by N-terminal sequencing analysis with corresponding reaction cycles.

data and the databases (Swiss-prot and *T. annulata*) data is a random event. The search results with scores derived from fragmented ion masses and corresponding identified proteins are calculated as follows:

MS/MS score = $-10(\log_{10}^{P})$, where P is the absolute probability that the observed match is a random event. For each search, a value is provided above which scores are significant at the 5% level ($P<0.05$). Protein scores are derived from ion scores on a non-probabilistic basis for the purpose of ranking protein hits.

Spot No.	Identified peptides with ion scores in brackets	Significant threshold ($P<0.05$)	Score of identified proteins	Identified proteins	Accession No.
TLS4	LVESLPQEIK (62), NYVQGINLVQAK (46), GEALAGAPLDNAPK (32)	45	157	Mitochondrial ribosomal protein L12 (MRPL12), bovine.	NP081480
TLS5	AQSELLGAADEATR (81), IEANEALVK (47)	44	128	ATP synthase-δ, mitochondrial precursor, bovine	P05630
TLS11	QTSGGPVDAGPEYQQDLDR (64)	45	64	ATP synthase 6, bovine	P13618
TLS12	VFLLGEEVAQYDGAYK (90), DFLIPLGK (46), TIRPMDIETIEASVMK (53), IMEGPAFNFLDAPAVR (86),	44	387	Pyruvate dehydrogenase-E1β (PDH E1-β), porcine	1917268B
TLS13	APAVTQHAPYFK (25), EISLDDFK (51), HLSVNDLPVGR (47), SVEETLR (33)	44	185	ATP-dependent proteinase SP-22, bovine	JC2258
TLS15	NPENTIFDAK (48), IINEPTAAAIAYGLDK (40), FEELCNEK (30), NQIFTTNEDR (30).	44	122	hsp70, *Theileria*	P16019
TLS16	AQYLGEPGTK (42), GKPLNEFLPNK (43)	22	96	putative inorganic pyrophosphatase, *Theileria*	Ta13735

Table 5.2 *T. lestoquardi* schizont associated proteins identified by Q-TOF analysis. The protein numbers corresponding to the spots in the 2D gel are as illustrated in Figure 4.6. Peptide sequences obtained from fragmented ions and identified proteins with scores are listed.

Twelve spots from 2DE were chosen for Q-TOF analysis and, from these, 7 proteins were identified. With the exception of TLS11, which yielded only one peptide sequence, two or more sequences were obtained from the fragmented ions derived from the spots analysed. The analysis led to the identification of proteins corresponding to TLS4, TLS5, TLS11, TLS12, TLS13, TLS15 and TLS16. The amino acid sequences of these proteins and their homologous alignments using the MegAlign Clustal V method of the DNASTAR programme are illustrated in Figures 5. 4 -10.

In total, 10 schizont associated proteins were identified by using MALDI-TOF, Q-TOF MS and N-terminal sequencing analysis (Table 5.3). *Theileria* hsp70 (TLS15, Fig. 5.9) and inorganic pyrophosphatase (TLS16, Fig. 5.10) were identified as putatively schizont secreted proteins and, although there are mammalian homologues existing in databases, the MS/MS facility of Q-TOF confirmed that TLS15 and TLS16 are *Theileria* proteins. TLS4 provided matches with bovine MRPL12 using both MALDI-TOF and Q-TOF analysis. Although two MRPL12 homologues have been found in *T. annulata* genome, they have low amino acid identity with bovine MRPL12 (17% and 20%, respectively, Fig. 5.4). Hence, TLS4 was identified as the ovine homologue of bovine MRPL12. An ATPase-δ homologue was also found in *T. annulata* genome. However, sequence alignment indicated that it has only 30% amino acid identity with bovine ATPase-δ and two peptide sequences revealed by Q-TOF were identical to the bovine sequence, confirming that TLS5 is ovine ATPase-δ (Fig. 5.5). Blast searching of the *T. annulata* genome identified a *Theileria* SP-22 homologous protein (TA17250, peroxiredoxin 1) and, although sequence alignment indicated 49% amino acid identity to the bovine homologue, Q-TOF MS/MS analysis identified it as bovine protein, confirming that TLS13 is ovine SP-22 (Fig. 5.8). Homologues of ATPase-6 and PDH E1-β were not found in the *T. annulata* or *T. parva* genomes, indicating that TLS11 and TLS12 are ovine ATPase-6 and PDH E1-β respectively (Fig. 5.6 and 5.7).

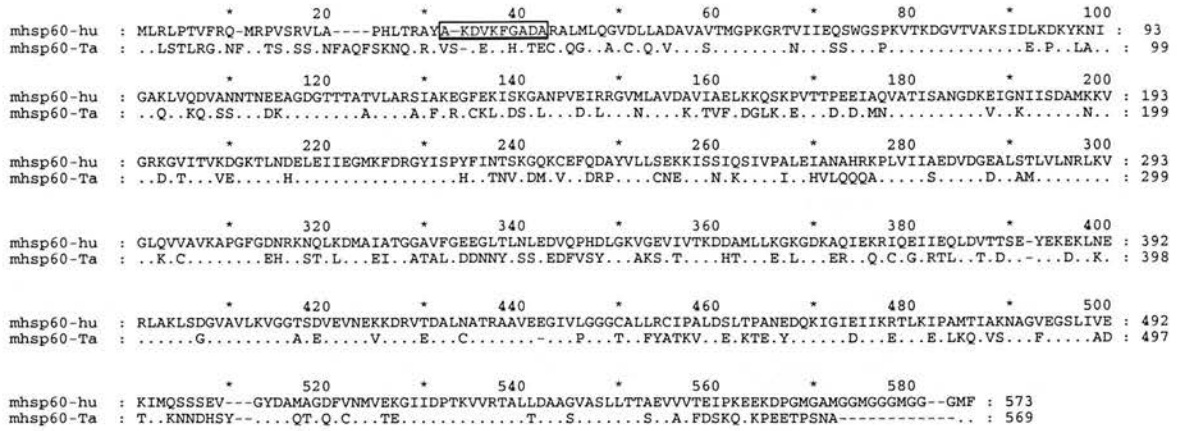


Fig. 5.2 Alignment of human mhsp60 (M34644) and its *T. annulata* homologue (TA07065). The boxed sequence represents the sequence that revealed by N-terminal sequencing of spots derived from 2D gels of schizont material.

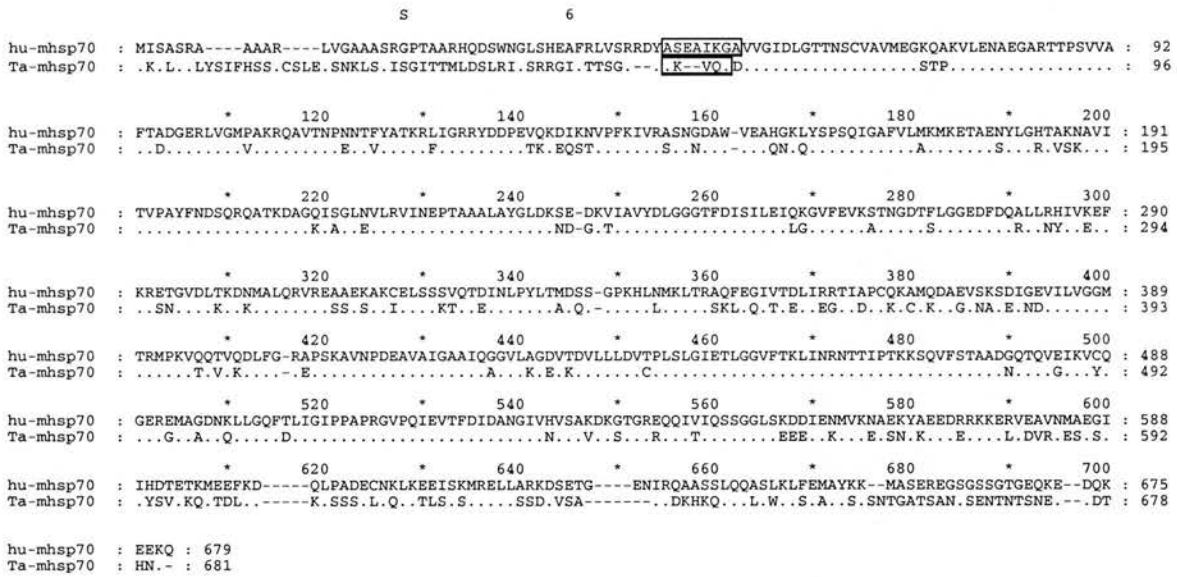


Fig. 5.3 Alignment of human (P38646) and *T. annulata* mhsp70 (TA14920) homologues. Boxed sequences represent those revealed by N-terminal sequencing of spots derived from 2D gels of schizont material.

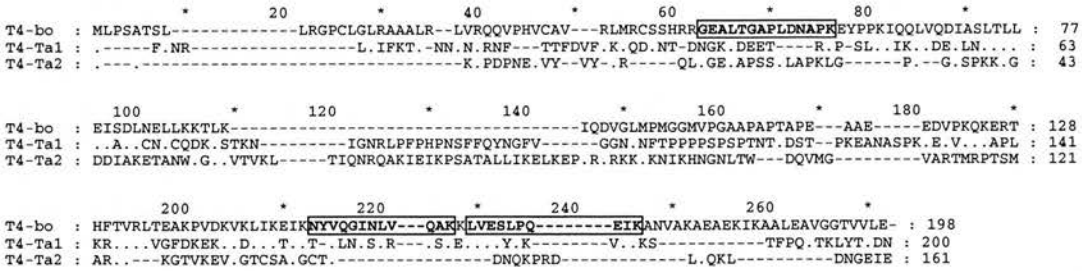


Fig. 5.4 Alignment of the bovine MRPL12 (NP_963900) amino acid sequence with those of two putative MRPL12 homologues (Ta15030 and TA07620) of *T. annulata*. Three peptide sequences revealed by Q-TOF MS/MS analysis are boxed.

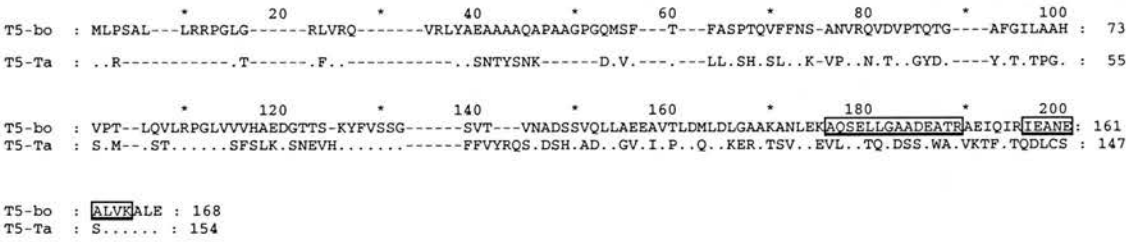


Fig. 5.5 Alignment of the bovine ATP synthase-δ (P05630) amino acid sequence with that of putative ATP synthase-δ homologue (Ta12155) of *T. annulata*. Two peptide sequences revealed by Q-TOF MS/MS analysis are boxed.

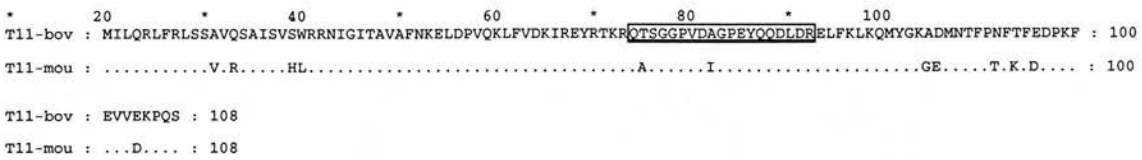


Fig. 5.6 Alignment of the bovine ATP synthase-6 (NP_777142) amino acid sequence with that of mouse homologue (P97450). One peptide sequence revealed by Q-TOF MS/MS analysis is boxed.

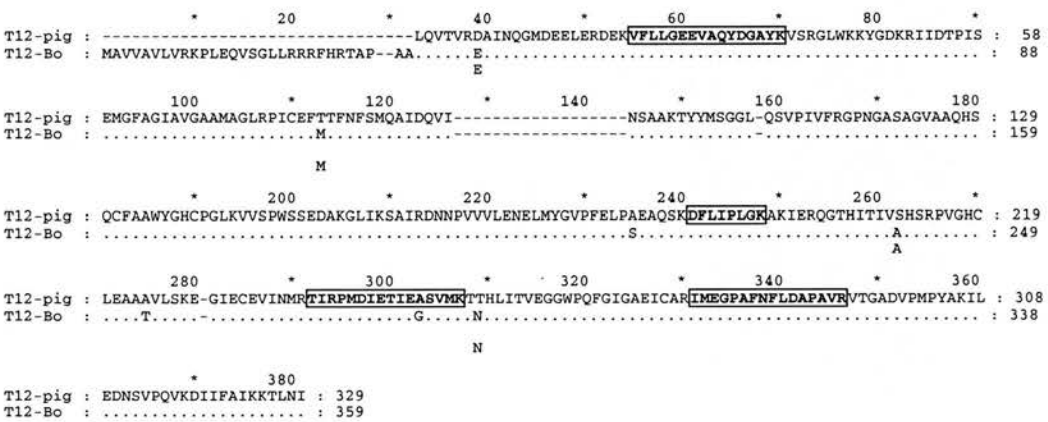


Fig. 5.7 Alignment of the porcine PDH E1-β (1917268B) amino acid sequence with that of bovine homologue (Tc206907). Four peptide sequences revealed by Q-TOF MS/MS analysis are boxed.

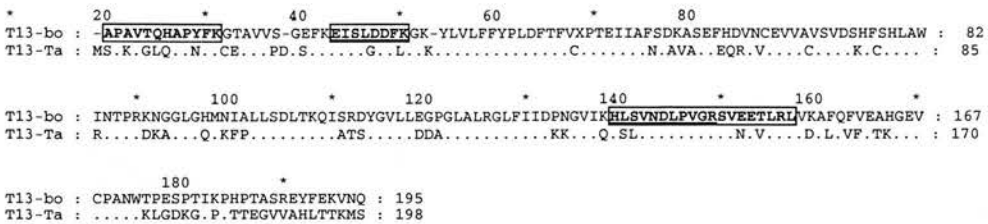


Fig. 5.8 Alignment of the bovine ATP-dependent proteinase SP-22 (JC2258) amino acid sequence with that of putative SP-22 homologue (TA17250) of *T. annulata*. Three peptide sequences revealed by Q-TOF MS/MS analysis are boxed.

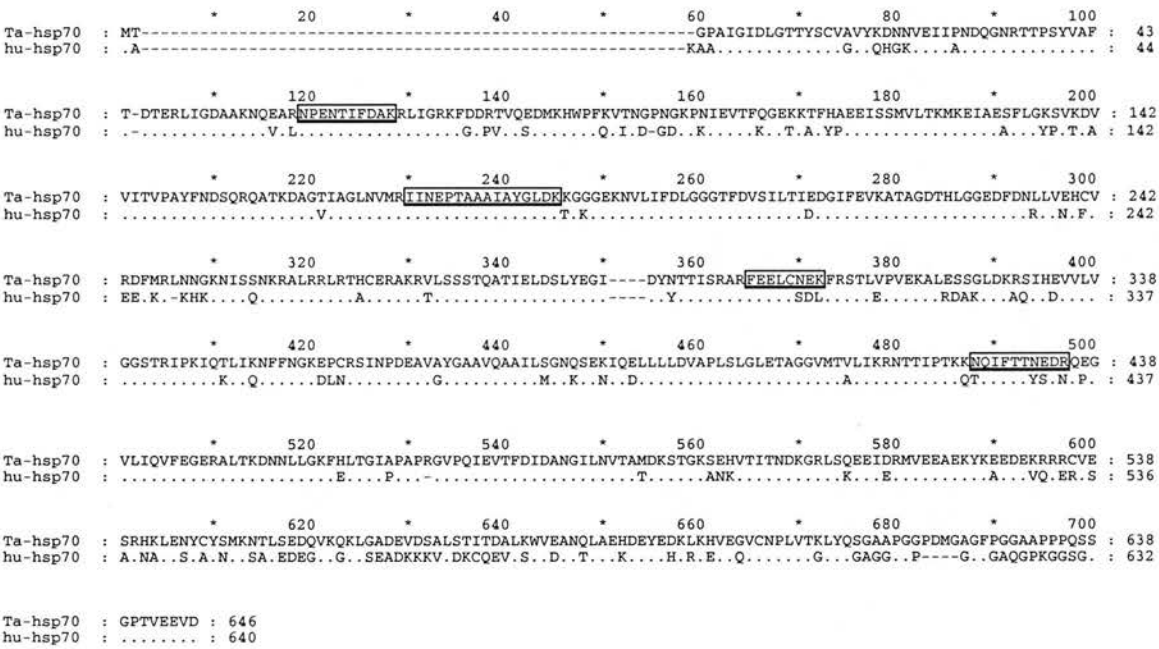


Fig. 5.9 Alignment of the *T. annulata* hsp70 (TA11610) amino acid sequence with the human hsp70 homologue (M11717). Four peptide sequences revealed by Q-TOF MS/MS analysis are boxed.

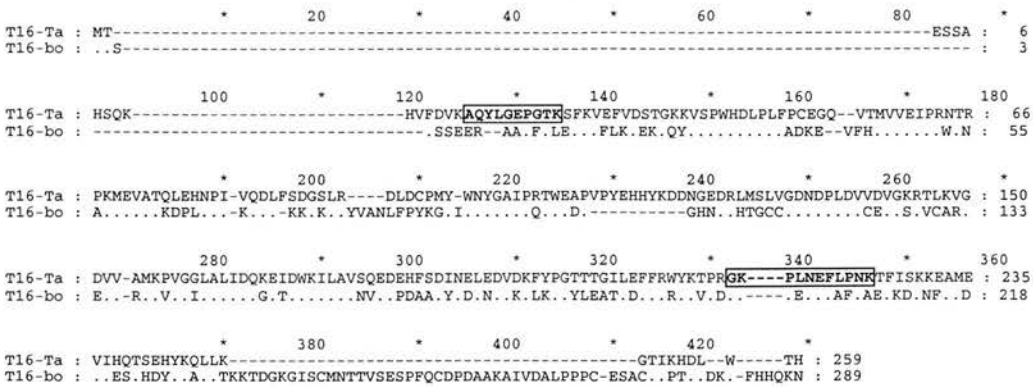


Fig. 5.10 Alignment of the *T. annulata* inorganic pyrophosphatase (Ta13757) with the bovine inorganic pyrophosphatase homologue (P37980). Two peptide sequences revealed by Q-TOF MS/MS analysis are boxed.

MALDI -TOF (Score)	Q-TOF (Score)	N-terminal sequence	Spot No.	Protein accession No.	Theoretical MW (kDa); pI	Observed MW (kDa); pI	Identified protein name	Protein identity
101	N/A	Yes	TLS1	P38646	74.0; 5.9	~75.0; ~5.9	Human mhsp70	Ovine homologue
103	N/A	Yes	TLS2	P10809	61.0; 5.7	~60.0; ~5.7	Human hsp60	Ovine homologue
56	N/A	Yes	TLS3	Ta14920	74.0; 5.8	~75.0; ~5.8	*T. annulata* mhsp70	*T. lestoquardi* homologue
63	122	No	TLS15	P16019	71.4; 5.3	~75.0; ~5.3	*T. annulata* hsp70	*T. lestoquardi* homologue
62	157	No	TLS4	NP081480	21.8; 9.3	~23.0; ~6.1	Bovine MRPL12	Ovine homologue
33	128	No	TLS5	P05630	15.1; 4.5	~16.0; ~4.5	Bovine ATP synthase-δ	Ovine homologue
40	64	No	TLS11	P13618	12.5; 5.4	~14.0; ~5.7	Bovine ATP synthase-6	Ovine homologue
53	387	No	TLS12	1917268B	36.1; 5.4	~35.0; ~5.85	Bovine PDH E1-β	Ovine homologue
N/A	185	No	TLS13	JC2258	21.7; 5.7	~22.0; ~6.2	Bovine SP-22	Ovine homologue
N/A	96	No	TLS16	Ta13735	29.6; 5.4	~32.0; ~5.85	*T.annulata* inorganic pyrophosphatase	*T. lestoquardi* homologue

Table 5.3 Summary of proteins identified from *T. lestoquardi* associated proteins by MALDI-TOF, Q-TOF MS and N-terminal sequencing analysis. Identified proteins are listed with accession number, theoretical mass and pI and individual scores obtained from MALDI-TOF and/or Q-TOF analysis. N/A indicates not applicable.

5.4 Discussion

As discussed in the previous chapter, 8 *Theileria* associated proteins were initially identified by MALDI-TOF analysis. These proteins were further analysed using either N-terminal sequencing or Q-TOF MS in the present study.

MALDI-TOF analysis and database searches revealed that TLS1 and TLS2 had strongest homology to human mhsp70 and hsp70 respectively, while TLS3 showed homology to a *T. annulata* mhsp70. Because of the conserved nature of hsps across species, these spots were subjected to N-terminal sequencing to confirm their identity. Eight and 10 amino acid residues were obtained from TLS1 and TLS2 respectively, although some gave only weak signals. After searching the database using the obtained amino acid sequences, TLS1 and TLS2 were again identified as human mhsp70 and hsp60 respectively (Fig. 5. 2 and Fig. 5.3), which confirms that TLS1 and TLS2 constitute the ovine homologues of these proteins.

Five amino acid residues from the TLS3 protein were determined by N-terminal sequencing and the protein was identified as Tamhsp70 by searching the *T. annulata* database (Fig. 5.3). This was consistent with the results from MALDI-TOF analysis (Table 4.1). The identity of TLS3 was therefore confirmed by two methods, MALDI-TOF MS and N-terminal sequencing, although both obtained relatively poor signals. The weak signals obtained from TLS1, TLS2, and TLS3 by N-terminal sequencing may be due to the low quantity of protein analysed. Although, theoretically, 1-100 picomoles of sample is required for sequencing by Edman degradation, practically, a minimum of 50 picomoles is required (Simpson 2003). One PVDF membrane blotted with schizont lysates was prepared in the present study and the amounts of TLS1, TLS2 and TLS3 protein provided for sequencing were estimated to be around 10-30 picomoles, which is below the minimum requirement. Provision of spots from more than one blotted PVDF membrane would increase protein amounts and possibly lead to a stronger signal.

Two or more residues were detected in some degradation cycles with each of the proteins (Table 5.1). This may have been caused by protein contamination or

mixtures of two proteins in one sample. Although in theory, a spot from a 2D gel represents an individual protein, the chance exists that two or more proteins (with similar pI and masses) could appear at same position and this would result in more than one amino acid residue obtained at some sequencing cycles. However, given that the majority of cycles yielded only one residue, it is likely that this ambiguity arose from the small quantity of protein provided.

Like N-terminal sequencing, Q-TOF can provide detailed sequence information and has been widely used for protein identification (Moormann et al. 1997). In a study using 2DE and mass spectrometry, 224 Coomassie blue stained spots were detected from *Haemonchus contortus* secreted proteins and, among them, 107 proteins were identified by MALDI-TOF analysis, with 62 of them were further confirmed by Q-TOF analysis of fragmented peptide ions. Q-TOF analysis has also identified novel proteins, such as cyclophilins, a nucleoside diphosphate kinase and microsomal peptidase H11, a vaccine candidate (Yatsuda et al. 2003). Using Q-TOF analysis and other tandem mass spectrometry methods, protein post-translational modifications, such as sulfonation on serine and threonine residues, were found in a cathepsin C like protein of *P. falciparum*. This finding suggests that sulfonation of serine and threonine may be involved in multiple functions, including protein assembly and signal transduction (Medzihradszky et al. 2004).

In the present study, TLS4, TLS5, TLS11, TLS12 and TLS15 were identified by Q-TOF analysis and gave more significant matches when compared with MALDI-TOF. TLS4, TLS5, TLS11 and TLS12 showed low scores and no significant matches by MALDI-TOF analysis, with scores of 62, 33, 40, and 53, respectively (significance threshold 63, $P<0.05$), In contrast, with the exception of TLS11, more than two-peptide sequences and high ion scores were obtained from each protein using Q-TOF. Database searches with this information provided matches for TLS4, TLS5, TLS11 and TLS12 proteins (Table 5.2). For TLS15, the *Theileria* hsp70, high scores were obtained from both MALDI-TOF and Q-TOF MS analysis (Table 4.1 and 5.2), which confirms the identity of TLS15 as *T. lestoquardi* hsp70. An additional *Theileria*

protein, TLS16, a putative inorganic pyrophosphatase, was also identified by Q-TOF and gave a significant identity match in the database search.

With the exception of TLS4, the theoretical pI and masses of the proteins identified in the databases were matched to pI and masses observed in 2D gels (Fig. 4.3) for each of the proteins identified by Q-TOF. The theoretical mass and pI of the bovine MRPL12, the strongest match for TLS4, are 21.8 kDa and 9.1, respectively. From 2D gels, it was found that the mass of the TLS4 spot is ~23.0 kDa and pI is ~6.1. The reasons behind the pI discrepancy are not clear. It is possible that they relate to degradation of the protein prior to 2DE. Alternatively, the differences may be associated with species-related variation between ovine MRPL12 and its bovine homologue. Confirmation of this possibility will require determination of the sequence of the ovine molecule.

Although Q-TOF has its advantages, such as high sensitivity with ability to provide sequence information, it still has its limitations. In Q-TOF analysis, some amino acids show similar fragmentation properties, such as leucine/isoleucine and glutamine/lysine, so that in some cases, it is difficult to analyse each combination of ion fragmentation patterns and to determine correct sequence information. Although Q-TOF is quicker than Edman degradation analysis, it basically provides peptide fragment ions rather than direct peptide sequences and therefore may not be precise enough to identify an unknown protein. Of the 12 spots analysed by Q-TOF, only 7 yielded sequence information leading to identification of a protein species. The remaining 5 spots were not identified and will need further analysis in future.

Using MALDI-TOF, Q-TOF and N-terminal sequencing in the present study, 3 schizont culture supernatant proteins of parasite origin were identified, namely *Theileria* mhsp70 (TLS3), hsp70 (TLS15) and inorganic pyrophosphatase (TLS16). A *T. parva* hsp70 (Daubenberger et al. 1997) and a *T. annulata* mhsp70 (Schnittger et al. 2000) have been described previously and their intracellular locations have been studied. *Theileria* inorganic pyrophosphatase is a novel protein but relevant

information can be obtained from other species. The potential functions of these putatively secreted schizont proteins are discussed further in chapter 7.

Apart from these schizont proteins, seven host derived proteins were identified (Table 5.2), namely: mhsp70 (TLS1); hsp60 (TLS2); mitochondrial ribosomal protein L12 (TLS4); ATP synthase-δ (TLS5); ATP synthase 6 (TLS11); PDH-E1β (TLS12); and ATP-dependent proteinase SP-22 (TLS13).

Mammalian mhsp70 belongs to the hsp70 family and is located mainly in the mitochondria. It plays a house-keeping role, acting as a molecular chaperone (Young 1990). Mammalian hsp60 was first identified as a tubulin-associated protein and was shown to be localized mainly in the mitochondrial matrix (Singh et al. 1990) and also present in extramitochondrial sites, including the cell surface and unidentified cytoplasmic vesicles (Gao et al. 1995). During infection with cellular pathogens, increased production of mhsp70 and hsp60 has been found to be essential for host cell survival and proliferation (Kaufmann et al. 1991). Both hsp 60 and hsp70 have also been found to act at key regulatory points in the control of apoptosis by directly interacting with different apoptotic proteins (Ravagnan et al. 2001; Xanthoudakis et al. 1999). Hisaeda et al. (1997) demonstrated that expression of hsp60 was associated with inhibition of apoptosis in *T. gondii*-infected cells. *T. parva* and *T. annulata* schizonts induce infected cells to undergo uncontrolled proliferation, which is similar to tumour cells and requires inhibition of apoptosis (Brown et al. 1973; Dobbelaere et al. 1999). Inhibition of apoptosis pathways in *T. parva* and *T. annulata*-infected cells has been well characterised and is described in detail in section 1.5, although a role for hsps in prevention of apoptosis in *Theileria*-infected cells has not been reported. It is possible, however, that the anti-apoptosis function of hsp60 and hsp70 observed in other pathogen-infected cells may occur in *Theileria*-infected cells.

Mammalian mitochondrial ribosomal protein L12 (MRPL12) is a member of a well characterized and conserved family of ribosomal proteins (Shah et al. 1997). It is encoded by nuclear genes, expressed in cytoplasm and transported into mitochondria (Mariottini et al. 1999). It also acts as a translational regulator of mitochondrial

mRNAs and dysfunction of MRPL12 activity upon expression of a negative dominant mutated protein causes reduced cell growth rate associated with the abolition of mitochondrial oxidative phosphorylation and ATP production (Marty et al. 1996; Marty et al. 1997). Depending on mitochondrial respiratory function, the pattern of MRPL12 expression is abundant in tissues such as heart, skeletal muscle, kidney which have a strong requirement for oxidative metabolism and less abundant in brain and liver which are less dependent on oxidative metabolism (Mariottini et al. 1999).

Pyruvate dehydrogenase-β (PDH-β) is a component of PDH complex (PDHC). The PDHC plays a key role in glucose metabolism by catalyzing the reaction of pyruvate with coenzyme A and forming CO_2, NADH and the intermediate acetyl CoA, which plays a central role in the citric acid cycle (CTA) and biosynthetic processes (Patel et al. 1990). Eukaryotic PDHC is located in the mitochondrial matrix and contains multiple copies of three enzymatic components: pyruvate dehydrogenase (PDH, E1), dihydrolipoamide acetyltransferase (E2) and lipoamide dehydrogenase (E3) (Patel et al. 1990).

ATPase coupling factor 6 (ATPase 6) and ATPase-δ belong to complex V of the oxidative phosphorylation pathway, which couples the passage of protons across the intermembrane space to the synthesis of ATP from ADP and Pi (Elston et al. 1998). ATPase consists of F0 and F1 components, which have 9 and 5 subunits respectively and constitute the catalytic core and the membrane proton channel (Walker et al. 1991). ATPase6, which is part of F0, forms a channel through which proton flow is coupled to rotation of the c-ring (Hutcheon et al. 2001). ATPase 6 protein has been isolated from bovine heart and porcine intestine and amino acid sequence comparison revealed that two homologues were highly conserved (Chen et al. 1987). ATPase-δ is one of the five polypeptides that constitute F1-ATPase and is part of the head unit of the enzyme. The function of the ATPase-δ is poorly defined. The gene of ATPase-δ has been cloned from bovine heart mitochondria and is 985 bp long and encodes a 22 amino acid mitochonrial import sequence before the mature protein N-terminal (Runswick et al. 1990).

ATP-dependent protease (SP-22) is a substrate protein of ATP-dependent proteinase, located in the mitochondrial matrix. It has been purified from bovine adrenal cortex and its amino acid sequence has been determined (Watabe et al. 1994). SP-22 was found to be 91% homologous to the MER5 protein of murine erythroleukemia cells, which is believed to play an important role in cell differentiation (Nemoto et al. 1990). SP-22 has also been found to be homologous to other proteins such as thioredoxin peroxidase in yeast (Chae et al. 1993) and the C22 component of an alkyl hydroperoxide reductase of *Salmonella typhimurium* (Tartaglia et al. 1990), both of which have antioxidant activity. Although the details of its biochemical function are unknown, it is suggested that SP-22 has a potential role in cell differentiation and also antioxidant activity (Watabe et al. 1995).

As outlined above, all of the host-derived proteins that were identified in schizont supernatants are of mitochondrial origin. In addition, five of these proteins (MRPL12, ATPase-δ, ATPase6, PDH E1-β and SP-22) are associated with the generation of ATP. This raised the possibility that their association with the schizont might be related to the parasite energy demands within the cell. Further investigation of the relationship of these proteins with the schizont are described in the following chapter.

Chapter 6: Investigation of the association of contaminating host proteins with the parasite

6.1 Introduction

The proteomic analysis of *T. lestoquardi* schizont preparations by MALDI-TOF, Q-TOF and N-terminal sequencing described in previous chapters identified host cell derived proteins including hsp60 (TLS2), MRPL12 (TLS4), ATPase-δ (TLS5), ATPase 6 (TLS11) and PDH E1-β (TLS12). Blast searching of the *T. annulata* and *T. parva* genomes using protein sequences of host derived MRPL12, ATPase-δ, ATPase 6 and PDHE1-β revealed a putative *Theileria* MRPL12 (Ta15030) and a putative ATPase-δ (Ta12155). However, ATPase-6 and PDHE1-β homologues were not identified. Since *Theileria* schizonts are obligate intracellular parasites (Brown 1983) and these proteins are important factors for energy production and cell growth (Chen et al. 1987; Mariottini et al. 1999; Walker et al. 1991), their presence in isolated schizonts might indicate that *Theileria* schizonts lack these factors and recruit them from host cells. This would fit with a model where the intracellular schizont recruits host mitochondrial proteins and these proteins are directly associated with schizonts.

An alternative hypothesis is that the identified host mitochondrial proteins are the result of host mitochondrial contamination in the schizont preparations. As described in section 2.3.4, EM was used to examine the purity of isolated schizonts and the results indicated that schizonts were present in large quantities and appeared with minimal host mitochondrial contamination. Theoretically, host mitochondria should have been separated from isolated schizonts by centrifugation through Nycodenz gradient media and three washes at 1900 × g (section 2.3.3). Mitochondria with normal size and density require 16, 000 × g for 15 min (Lodish 1999) or 13, 000 × g for 1 hr (Sinai et al. 1997) to form the pellet and would not be expected to sediment at lower centrifugal forces. If there was still significant mitochondrial contamination in the schizont preparations, it would suggest that there is a close association between the schizonts and the host mitochondria. No study of the association of

schizonts with mitochondria has been reported in *Theileria*-infected cells. However, a tight association of host microtubules and *Theileria* parasites has been observed (Fawcett et al. 1984; Shaw et al. 1991). It is theoretically possible that host cell microtubules are also closely associated with host cell mitochondria, thereby bringing parasite and mitochondria into close proximity.

Immunofluorescence has been widely used to identify the cellular or subcellular locations of proteins (Neri et al. 1992). Immunofluorescence assays can be direct or indirect. The latter is based on the specific interaction of a primary antibody with its target antigen inside a chemically-fixed and permeabilized cell. The antigen-antibody complex is visualised using a fluorescent-tagged secondary antibody that recognizes the primary antibody. Such immuno-fluorescence analysis (IFA) can be extended to localize more than one protein in the same sample by using different secondary antibodies conjugated to distinct fluorochromes. Multi-labelling of the same sample can be analysed by confocal microscopy. Confocal microscopy, a light microscopical imaging technique introduced by M. Petran and A. Boyde in the 1980s, can produce optical sections through a 3-dimensional specimen and allows the operator to colocalise more than one antigen in the same sub-cellular organelle (Coleman et al. 2000).

Dual-labelling and confocal microscopy has been used to localize intracellular distribution of novel proteins in *T. parva*-transformed cells (Heussler et al. 2002). Using antibodies directed against PIM, a *T. parva* schizont surface protein, in combination with antibodies against subunits of the host derived IKK complex, it was shown that IKK is located on the surface of *T. parva* schizonts and remains associated with schizonts throughout the host cell interphase (Heussler et al. 2002).

This chapter describes the use of a mitochondrial probe and dual-labelling immunofluorescence analysis and confocal microscopy to investigate the localisation of host mitochondrial proteins within the infected cells.

6.2 Materials and methods

6.2.1 Immunofluorescence analysis

6.2.1.1 Cell samples

TLL maintained in the lab were harvested when the cells were in their exponential growth phase and resuspended in complete IMDM (5×10^5 cells/ml) as described in section 2.3.

6.2.1.2 Antibodies

Host derived proteins of hsp60 (TLS2), MRPL12 (TLS4), ATPase-δ (TLS5), ATPase 6 (TLS11) and PDH E1-β (TLS12) were chosen to investigate their intracellular localisation.

Primary antibodies: mouse anti- human hsp60 mAb (IgG1) was purchased from Stressgen Biotechnologies (Canada); mouse anti-human PDH E-β mAb (IgG1) was purchased from Molecular Probes Inc. (Netherlands). Other primary antibodies against TLS4, TLS5, TLS11 and TLS12 were commercially unavailable and raised in rats as described below. A rabbit polyclonal antibody (SA), raised against the C – terminal of the Tamhsp70 (Schnittger et al. 2000), was kindly provided by Prof. Jabbar Ahmed, Germany. A mouse mAb, IC12 (IgG1), raised against a *T. annulata* schizont surface protein, was kindly provided by Dr Brian Shiels, Glasgow University.

Secondary antibodies: FITC-conjugated goat anti-rat IgG, FITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG were purchased from Sigma (USA). Rhodamine red-X (RRX)-conjugated Fab fragments of goat anti-mouse IgG and RRX- Fab goat anti-rabbit IgG were purchased from Jackson Immunoresearch Laboratories (USA).

6.2.2 Generation of rat polyclonal antisera

TLS4, TLS5, TLS11 and TLS12 proteins are all host mitochondrial proteins that copuried with isolated schizonts. These were abundant in 2DE gels (Fig. 4.6) and were therefore chosen for raising polyclonal antibodies in rats.

Schizont proteins were separated by 2DE as described in section 4.2 and 4.3. Total *T. lestoquardi* schizont proteins (40 μg per 7 cm IPG strip) were resolved in the first dimension and three IPG strips (7 cm, nonlinear, Amersham Biosciences) were placed on the ExcelGel SDS gel (250 × 110 × 0.5 mm, Amersham Biosciences). Spots corresponding to TLS4, TLS5, TLS11 and TLS12 were excised on the basis of individual mass and pI. To increase the amount of protein for rat immunisation, each spot was excised from 3 gels, cut into small pieces (approximately 0.5-1 mm in diameter), pooled and homogenised in 300 μl PBS using a syringe and needle (26G, 0.45 ×12 mm, Terumo). Quil A adjuvant (10 μg, Brenntay Biosector, Denmark) was then added to 300 μl of each suspension. One hundred microlitres of each suspension was then injected subcutaneously into each of three female rats (Wistar strain, 10 weeks old) on three occasions at four week intervals.

Rats were bled (~50 μl) prior to immunisation for preparation of control serum and before each boost to monitor antibody levels. Four weeks after the second boost, all immunised rats were killed and their blood (1-1.5 ml for each rat) was collected individually in 1.5 ml eppendorf tubes. Blood was allowed to clot for 30-60 min at 37 °C and the clot was separated from the sides of the tube using a Pasteur pipette. The tube was then placed at 4 °C overnight to allow the blood clot to contract. The serum was transferred into an eppendorf tube and centrifuged at 10,000 rpm for 5 min in a microfuge at 4 °C to remove any remaining insoluble material. Serum was then collected and stored in 1.5 ml eppendorf tubes at -20 °C.

6.2.3 Screening of rat sera

To investigate recognition of specific antigens in TLL, rat sera were initially tested using conventional IFA on TLL. Smears of TLL were prepared on 15-well culture multi-test slides (ICN Ltd) as described in section 2.4. Five microlitre aliquots of TLL (5×10^5 cells/ml) were dropped onto each well of the slide, allowed to dry, and fixed in cold methanol. Antiserum from each rat was diluted in PBS at 1:20, 1:40 and 1: 80 and 10 µl of each dilution was added to individual wells, incubated for 30 min at RT, washed with PBS for 3 times and followed by10 µl of FITC -conjugated goat anti-rat IgG (Sigma) at 4 µg/ml in PBS for 30 min. Identical dilutions of pre-immune serum from each rat were used as controls. TLL labelling was observed using a BX50 fluoresent microscope (Olympus Optical Co Ltd).

6.2.4 Optimisation of single-labelling of TLL for confocal microscopy analysis

Conditions for cell staining with rat antisera raised against TLS4, TLS5, TLS11 and TLS12 (named TLS4 serum, TLS5 serum, TLS11 serum and TLS12 serum, respectively) required further optimisation for confocal microscopy analysis. Mouse mAb (IC12) was raised against the *T. annulata* schizont surface protein and had not previously been tested against *T. lestoquardi* parasites. IC12 was initially tested in TLL to see whether it labelled the *T. lestoquardi* schizont surface. Subsequently, titrations of IC12 and SA against *T. annulata* mitochondria hsp70 were optimised and used to locate *T. lestoquardi* schizont in infected cells by dual-labelling and confocal microscopy analysis.

6.2.4.1 TLL cytospin preparations

TLL were resuspended in complete IMDM at a density of 5×10^5cells/ml and aliquots of 50 µl were used to prepare cytospin smears by centrifugation at 600 rpm for 5 min in a cytospin centrifuge (Thermo Shandon, UK). Slides were allowed to air-dry and fixed for 15 min at RT in 3.7% formaldehyde diluted in PBS. Slides were then washed in PBS three times, permeabilized in ice-cold acetone for 5 min, and washed in PBS three times.

Unless stated otherwise, all labelling procedures were performed at 21°C under humidified conditions using the Sequenza immunostaining system (Thermo Shandon, UK). This uses the Coverplate system which ensures consistency and higher staining quality than a humidified chamber. It also allows the user to stain large batches of slides (up to 50 slides/run) with a low chance of batch variation.

6.2.4.2 TLL single labelling analysis

To reduce background and minimize cross-reactivity, slides were rinsed and incubated for 1 hr at RT in blocking buffer (0.5 M NaCl in PBS, pH 7.4) containing 10% normal serum from the same species as the secondary antibody. Dilutions of primary antibody or normal control serum (100 µl each) were then applied to the cytospin preparations and these were incubated overnight at 4 °C. Slides were washed 3 times in PBS for 5 min and incubated for 1 hr at RT with 100 µl FITC-conjugated secondary antibodies, goat anti-rat/mouse/rabbit IgG (depending on species of the first antibody, Sigma) diluted at 4 µg/ml in blocking buffer. Primary and secondary antibodies with individual dilutions are listed in table 6.1. Slides were then washed 3 times with PBS before the addition of 10 µl DRAQ5 (Biostatus Ltd, Alexis corporation, Switzerland) diluted at 1:1000 in PBS for a further 5 min. Slides were finally washed 3 times with PBS, dried and mounted in Mowiol (pH 8.5) mounting media (EMD Biosciences, US) under 1.5 (0.17 mm thickness) glass coverslips (BDH Laboratory Supplies).

Fluorescent images were acquired using an MRC-600 confocal laser scanning microscope (CLSM: Bio-Rad Laboratories, UK) mounted on an Axiovert 100 inverted microscope equipped with a 1.4 NA (x63) Plan-Apochromat® objective lens (Carl Zeiss, Welwyn Garden City, U.K.). Fluorophores were excited and imaged sequentially using the 488 nm (FITC), 568 nm (Rhodamine Red-X: RRX) and 647 nm (DRAQ5) lines from a 15 mW Kr/Ar laser (Bio-Rad Laboratories). Images were prepared using Photoshop (Adobe Systems, UK).

Primary antibodies	Secondary antibodies
Rat sera (TLS4, 5 and 12) and corresponding pre-immune serum at 1: 50, 1:100, 1:150 and 1:200 dilutions	FITC-conjugated goat anti-rat IgG (4 μg/ml)
SA/normal rabbit serum at 1:250, 1:500 and 1:1000 dilutions	FITC-conjugated goat anti-rabbit IgG (4 μg/ml)
IC12/normal mouse IgG1 at 1:10, 1:20 and 1:40 dilutions	FITC-conjugated goat anti-mouse IgG (4 μg/ml)
PDH E1β mAb at 8, 4, 2 μg/ml	
Hsp60 mAb at 8, 4, 2 μg/ml	

Table 6.1 Primary and secondary antibodies used in single labelling for confocal microscopy.

6.2.5 Dual labelling of TLL for analysis by confocal microscopy

TLL cytospins were prepared as described above for single labelling. The first primary antibodies (100 μl at optimal dilutions in blocking buffer) were added to the slides and incubated overnight at 4°C. Slides were then washed once in blocking buffer for 5 min and incubated for 1 hr at RT with 100 μl goat anti-rat/mouse IgG FITC-Fab fragments at 4 μg/ml diluted in blocking buffer. Slides were then washed once in blocking buffer.

After labelling with the first primary antibody, slides were incubated in blocking buffer for 1 hour at RT and then incubated for 2 hrs at RT with 100 μl of the second primary antibody (SA or IC12) diluted in blocking buffer. Slides were washed 3 times in PBS and incubated for 1 hr at RT with 100 μl goat anti-mouse/rabbit IgG Rhodamine Red-X (RRX) -Fab fragments at 4 μg/ml in blocking buffer. After washing with PBS 3 times, slides were mounted and the distribution of different fluorescent labels was observed by confocal microscopy.

The primary antibodies and fluorescent secondary antibodies used in the dual-labelling are listed in table 6.2. To minimise cross-reactivity in dual labelling of TLL

with two primary antibodies from the same species, such as PDH E1β mAb-IC12, hsp60 mAb-IC12, PDH E1β-hsp60 mAbs, after first primary and secondary labelling, the second primary antibody and secondary goat anti-mouse IgG RRX-Fab fragments were reacted at RT for 15 min to form immuno-complexes and blocked with 10% normal mouse serum for 20 min prior to addition to TLL.

First primary Ab	Secondary Ab	Second primary Ab	Secondary Ab
TLS4 serum or preimmune serum (1:150)	FITC- Fab of goat anti-rat IgG (4 μg/ml)	SA/normal rabbit serum (1:1000) OR IC12/mouse IgG1 (1:20)	SA secondary: RRX- Fab of goat anti-rabbit IgG (4 μg/ml) OR IC12 secondary: RRX-Fab of goat anti-mouse IgG (4 μg/ml)
TLS5 serum or preimmune serum (1:100)	As above		
TLS12 serum or preimmune serum (1:100)	As above		
PDH E1β mAb/mouse isotype matched IgG1 (2 μg/ml)	FITC-Fab of goat-mouse IgG (4 μg/ml)		
Hsp60 mAb/mouse isotype matched IgG1 (2 μg/ml)	As above		
TLS4 serum or preimmune serum (1:150)	FITC- Fab of goat anti-rat IgG (4 μg/ml)	Hsp60 mAb/matched isotype control (2 μg/ml)	IC12 secondary: RRX-Fab of goat anti-mouse IgG (4 μg/ml)

Table 6.2 Combinations of primary and secondary antibodies used in dual-labelling with optimum dilutions in parentheses.

6.2.6 Analysis of host mitochondrial contamination using MitoTracker dye

MitoTracker dye, a mitochondrial-selective probe, was initially used to investigate host mitochondrial contamination of isolated schizonts.

To determine the optimal staining concentration, MitoTracker dye was first tested by staining TLL and sheep PBMC blasts. TLL maintained in the lab were harvested in their exponential growth phase and resuspended in complete IMDM (2×10^6 cells/ml)

as described in section 2.3. Sheep PBMC (2×10^7) were prepared as described in section 2.1 and cultured with concanavalin A (ConA, Sigma) at a final concentration of 10 μg/ml in complete IMDM for 20 hrs. The resultant cell blasts were harvested at $500 \times g$ for 10 min and resuspended at 2×10^6 cells/ml in complete IMDM.

For live-cell staining, the recommended working concentration for MitoTracker red 580 dye (Molecular Probes) is between 25-500 nM. Samples of TLL and PBMC blasts at 2×10^6 cells/ml were dispensed into 1.5 ml Eppendorf tubes (50 μl each) and MitoTracker dye of 1 mM stock solution in dimethylsulfoxide (DMSO) was diluted to 1μM in complete IMDM and added into each cell suspension to give final working concentrations of 50 nM, 100 nM, 200 nM, 300 nM and 400 nM. They were incubated for 20 mins at 37 °C, pelleted by centrifugation at 10, 000 rpm in a microfuge centrifuge (Microfuge®, Beckman) for 3 min and resuspended in fresh prewarmed complete IMDM. Five microlitre aliquots of cell suspension were dropped on to each well of the 15 well-slides (ICN Ltd) and fixed in 3.7% formaldehyde in complete IMDM at 37 °C for 15 min. Slides were rinsed in PBS for 3 min, permeabilised in ice-cold acetone for 5 min, rinsed again in PBS for 3 min and then observed under a BX50 fluorescence microscope.

Isolated schizonts at 2×10^6 cells/ml were subsequently stained with an optimal concentration of MitoTracker dye and observed as described above. To further investigate any association of MitoTracker staining with *T. lestoquardi* schizonts in TLL, the *Theileria* specific antibody SA was used to label TLL after staining with MitoTracker dye. Slide smears of TLL stained by MitoTracker dye as described above, after fixation and permeabilization, were incubated with 10 μl of SA at 1:400 in PBS for 30 min at RT followed by 10 μl of FITC-conjugated goat anti-rabbit IgG (1:100 in PBS) for 30 min and observed under the fluorescent microscope.

6.2.7 Electron microscopy

The ultrastructural morphology of TLL was examined by EM and compared with that of PBMC blast cells derived by treatment with ConA as described in section

6.2.6). TLL (5×10^7 cells) were harvested in their exponential growth phase and sheep PBMC blast cells were prepared as described above. Cells were pelleted by centrifugation at $350 \times g$ for 5 min, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH7.3) and stained with uranyl acetate and lead citrate as described in section 2.2.5.3. Cell ultrastructure in ultra-thin sections was observed under a Jeol 1010 electron microscope operating at 80 KV.

6.2.8 Dual-labelling of isolated schizonts and confocal microscopy analysis

To evaluate host mitochondrial contamination in isolated schizont preparations, schizont specific antibodies, IC12 and SA, were used along with host mitochondria-specific antibody, hsp60 mAb, to dual-label schizonts. Five microlitre aliquots of schizonts isolated from 2×10^7 TLL as described in section 2.2 were dropped onto the slides, allowed to dry, fixed and dual-labelled with the hsp60 mAb and IC12 or SA as outlined in table 6.3 and observed by confocal microscopy as described in section 6.2..

First primary Ab	Secondary Ab	Second primary Ab	Secondary Ab
Hsp60 mAb/mouse isotype matched IgG1 (2 μg/ml)	FITC-Fab of goat-mouse IgG (4 μg/ml)	SA/normal rabbit serum (1:1000)	RRX- Fab of goat anti-rabbit IgG (4 μg/ml)
		IC12/mouse isotype matched IgG1 (1:20)	IC12 secondary: RRX-Fab of goat anti-mouse IgG (4 μg/ml)

Table 6.3 Primary and secondary antibodies used in dual-labelling of isolated schizonts.

6.3 Results

6.3.1 IFA analysis

6.3.1.1 IFA screening of rat antisera

Rat sera were collected before each inoculation and tested by IFA on TLL. Four weeks after the second inoculation, one rat from each group immunised with TLS4 and TLS5 spots showed positive labelling compared to the preimmune serum control. Four weeks after the third inoculation, TLL were positively labelled by the antiserum of at least one rat in each group. Results of the rat serum testing are summarised in table 6.4.

		Rat antibody responses 4 weeks after each inoculation		
Group	Preimmune sera	1st inoculation	2nd inoculation	3rd inoculation
TLS4	–	–	+ (1/3)	+ (2/3)
TLS5	–	–	+ (1/3)	+ (2/3)
TLS11	–	–	–	+ (1/3)
TLS12	–	–	–	+ (1/3)

Table 6.4 TLL IFA analysis with rat sera (1:40) collected 4 weeks after successive inoculations with TLS4, 5, 11 and 12 proteins. + indicates positive labelling and – indicates negative labelling. Figures in parentheses represent numbers of rats reacting.

Two rat antisera from each of the TLS4 and TLS5 inoculated groups showed positive labelling of TLL at a 1: 40 dilution when compared with preimmune sera controls (Fig. 6.1, panel A, B). The two antisera showed strong background at 1:20 and weak staining at 1:80 (data not shown). TLS11 and TLS12 antisera showed TLL positive staining at 1: 40 compared to the preimmune controls (Fig 6.1, panel C, D) and very weak labelling at 1:80 dilution (data not shown). This preliminary analysis confirmed that TLS4, TLS5, TLS11 and TLS12 sera recognize proteins in TLL.

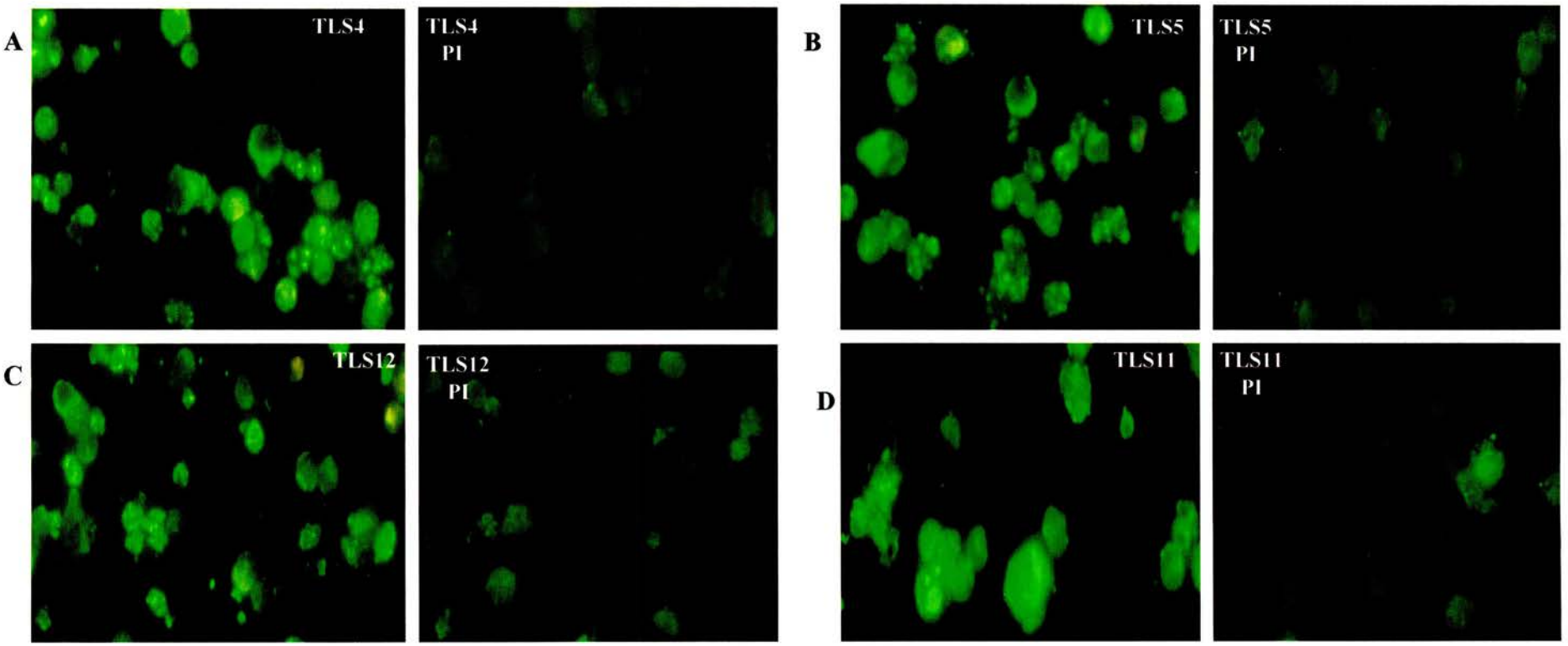


Fig. 6.1 IFA of TLL treated with polyclonal rat sera

TLL were labelled with rat sera raised against TLS4 (panel A), TLS5 (panel B), TLS12 (panel C) and TLS11 (panel D) and the respective pre-immune (PI) controls at 1:40 dilution, followed by secondary goat anti-rat FITC-IgG at 1:100 dilution. Pictures were taken at x 200 magnification.

6.3.2 Confocal images from optimised TLL single labelling

DRAQ5 is a cell-permeable anthraquinone that allows rapid staining of nuclear DNA in live or fixed mammalian cells (product information, Biostatus Ltd). It was used to counterstain TLL after labelling with TLS4, TLS5 and TLS12 sera, and the hsp60 mAb. TLL labelling with DRAQ5 showed that it specifically stained nuclei of both host cell and schizonts. The schizont nuclei were easily distinguished from the host cell nuclei allowing location of the schizonts within TLL. Figure 6.2 to Fig. 6.6 illustrate that a single large host cell nucleus appears in each cell in contrast to the smaller multinucleated schizonts. It is also apparent that more than one schizont was present in some of the host cells (arrows).

TLS4 serum was evaluated at dilutions of 1:50, 1:100, 1:150 and 1:200. Although labelling of TLL at 1:200 dilution was weak (data not shown), specific labelling was observed at 1:150 with significant improvement in background staining (Fig. 6.2A) as compared to that seen at 1: 40 dilution (Fig. 6.1A). Labelling was most intense around the schizonts, although some was also apparent in the host cell cytoplasm (Fig. 6.2). TLS5 serum showed weak labelling at 1:150 and 1:200 dilutions (data not shown). At 1:100 dilution, TLS5 serum labelled the area around the schizonts, as well as some labelling in the cytoplasm (Fig. 6.3). Preimmune sera showed no labelling in either case.

TLS12 serum showed weak labelling at 1:150 and did not give any labelling at 1:200 (data not shown). At 1:100 dilution, it demonstrated similar labelling to TLS5 serum. However, corresponding preimmune serum showed some staining of TLL cytoplasm (Fig. 6.4), suggesting that non-specific labelling occurred. A commercial PDH E1β mAb was therefore obtained and subsequently used to detect the protein in TLL. This antibody was evaluated at dilutions of 2, 4 and 8 μg/ml and at 2 μg/ml showed a staining pattern in TLL consistent with a mitochondrial location (Fig. 6.5). The hsp60 mAb was also evaluated at dilutions of 2, 4 and 8 μg/ml and at 2 μg/ml labelled the host cell cytoplasm with staining being most intense in the region occupied by schizonts. The control antibody in each instance showed no staining (Fig. 6.6).

The modified staining protocol based on longer incubation period and optimal antibody dilution significantly reduced background fluorescence and improved the specificity of labelling. These optimised labelling conditions were subsequently used for dual-labelling and confocal microscopy analysis.

6.3.3 Confocal images with TLL dual-labelling

6.3.3.1 Dual-labelling of TLL with *T. annulata* specific antibodies

To determine whether TLS2, TLS4, TLS5 and TLS12 proteins were associated with schizonts in TLL, SA, a rabbit antiserum raised against *T. annulata* mitochondrial hsp70 and IC12, a mAb that recognises a *T. annulata* surface protein, were used in conjunction with the protein-specific antibody reagents. For confocal studies, SA was tested at dilutions of 1:250, 1:500 and 1:1000. All gave good schizont labelling, with staining locating to the parasite mitochondria (Fig. 6.7A).

IC12 had not been previously tested on *T. lestoquardi* and was evaluated at dilutions of 1:10, 1:20 and 1:40. Labelling was weak at 1:40 (data not shown) and, at 1:20, good staining of the schizont surface was observed (Fig. 6.7B). Dual-labelling of TLL with SA and IC12 clearly defined the boundary of the schizont surface and the internal location of the parasite mitochondria (Fig. 6.7C).

6.3.3.2 Dual-labelling of TLL and confocal microscopy analysis

Dual-labelling of TLL with TLS4 serum and SA revealed that the TLS4 serum appeared to label the schizont as well as the host cell, where labelling was consistent with its location in mitochondria (Fig. 6.8). Some overlapping of the two labels as indicated by yellow staining was observed in schizonts, which suggests that the two proteins may be co-localised within parasite mitochondria. Dual labelling of the TLS4 control serum (TLS4 preimmune) with SA and TLS4 serum with the SA control (normal rabbit serum) showed no overlapping of labels (Fig. 6.8). These observations were supported by dual-labelling of TLL with TLS4 serum and IC12

which also suggested the presence of TLS4 within the schizonts (Fig. 6.9). Control antibodies showed clear background (Fig. 6.9).

Dual-labelling of TLL with TLS5 serum and SA showed a diffuse granular pattern of staining in the host cell cytoplasm and no co-localisation with SA was observed (Fig. 6.10). TLS5 serum dual-labelling with IC12 showed that TLS5 staining appeared concentrated in the area surrounding the schizonts (Fig. 6.11).

Dual labelling of TLL with the PDH E-β mAb and IC12 showed a granular pattern of staining in host cell cytoplasm, with no evidence of co-localisation with IC12. Some mitochondria-like structures were apparent and, again, staining appeared more intense in the region occupied by the schizont (Fig. 6.12).

Dual-labelling of TLL with hsp60 mAb and SA, along with nuclear staining by DRAQ5 revealed a hsp60 mAb staining pattern consistent with a mitochondrial location, with dense aggregates in the region of the schizont in some cells (Fig. 6.13A). Controls of hsp60 mAb and SA showed no cross reactivity (Fig. 6.13B and C). Dual labelling of TLL with hsp60 and IC12 mAbs showed a similar pattern (Fig.6.14) and confirmed that host hsp60 is not associated with the schizont, either internally or on its surface.

Dual-labelling of TLL with hsp60 mAb and TLS4 serum showed co-localization in host cell cytoplasm with staining being consistent with a mitochondrial distribution for both proteins. Organelles labelled only with TLS4 serum were also observed and probably represent schizont mitochondria (Fig. 6.15). Some organelles were also observed that labelled only with hsp60 mAb.

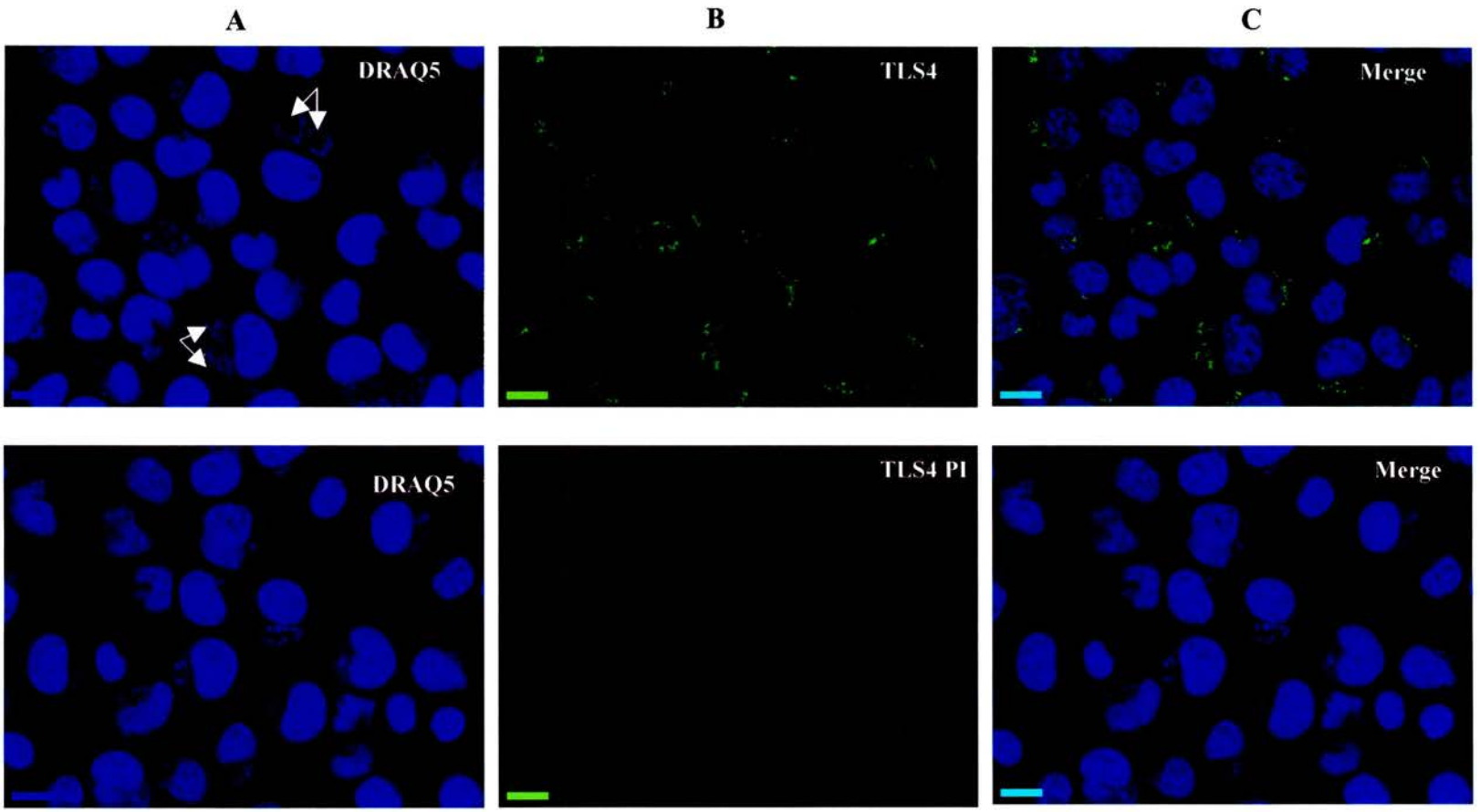


Fig. 6.2 Confocal images of TLL labelled with TLS4 serum and DRAQ5

TLL were labelled with TLS4 serum (top) or preimmune serum (bottom) at 1:150, followed by goat anti-rat FITC-IgG (4 μg/ml) and DRAQ5 diluted at 1:1000. A, DRAQ5; B, TLS4 serum/preimmune serum; C, merged images. Arrows indicate multiple schizonts in TLL. Bars, 10 μm.

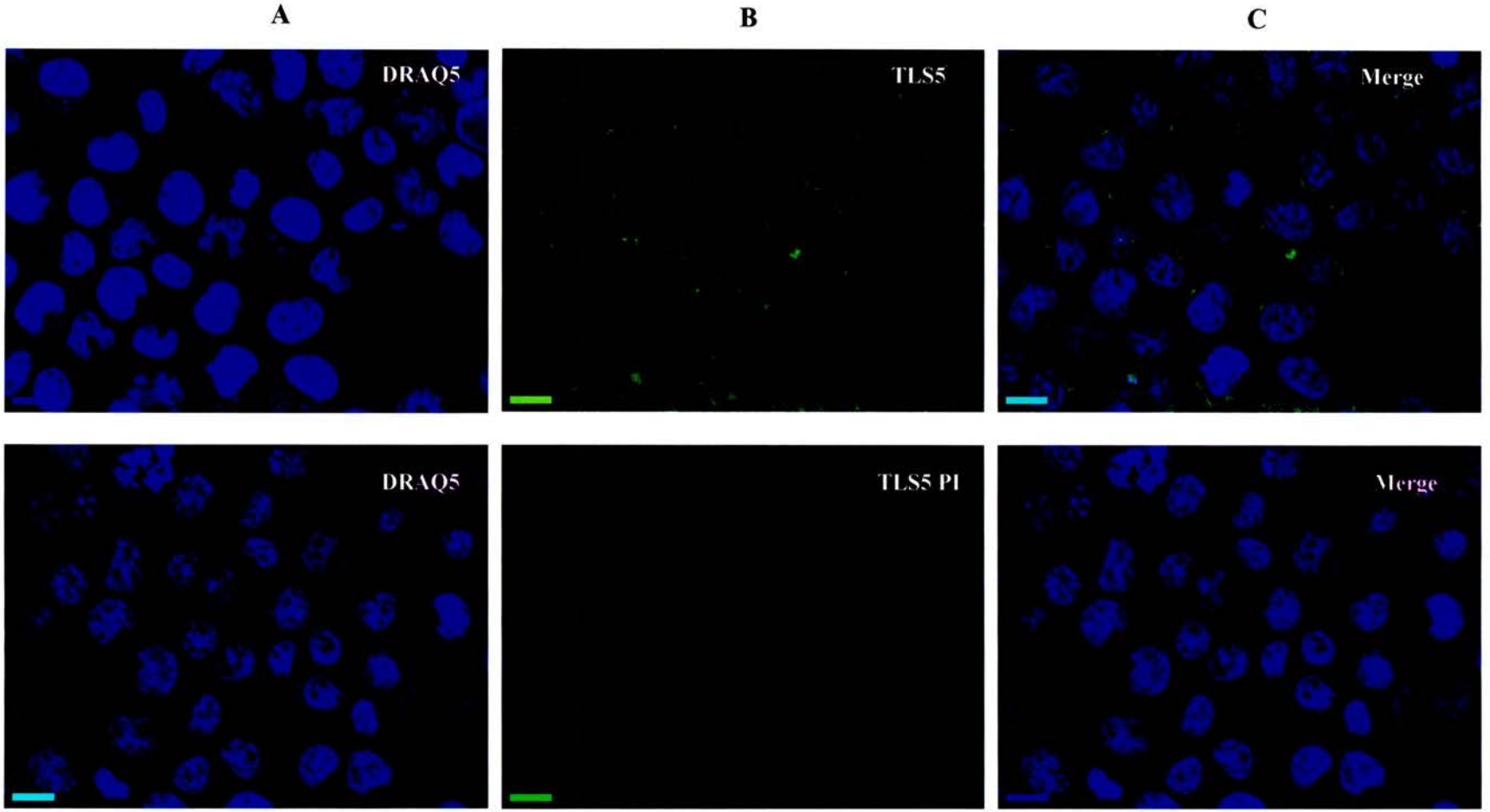


Fig. 6.3 Confocal images of TLL labelled with TLS5 serum and DRAQ5

TLL were labelled with TLS5 serum (top) or preimmune serum (bottom) at 1:150, followed by goat anti-rat FITC-IgG (4 μg/ml) and DRAQ5 diluted at 1:1000. A, DRAQ5; B, TLS5 serum/preimmune serum; C, merged images. Bars, 10 μm.

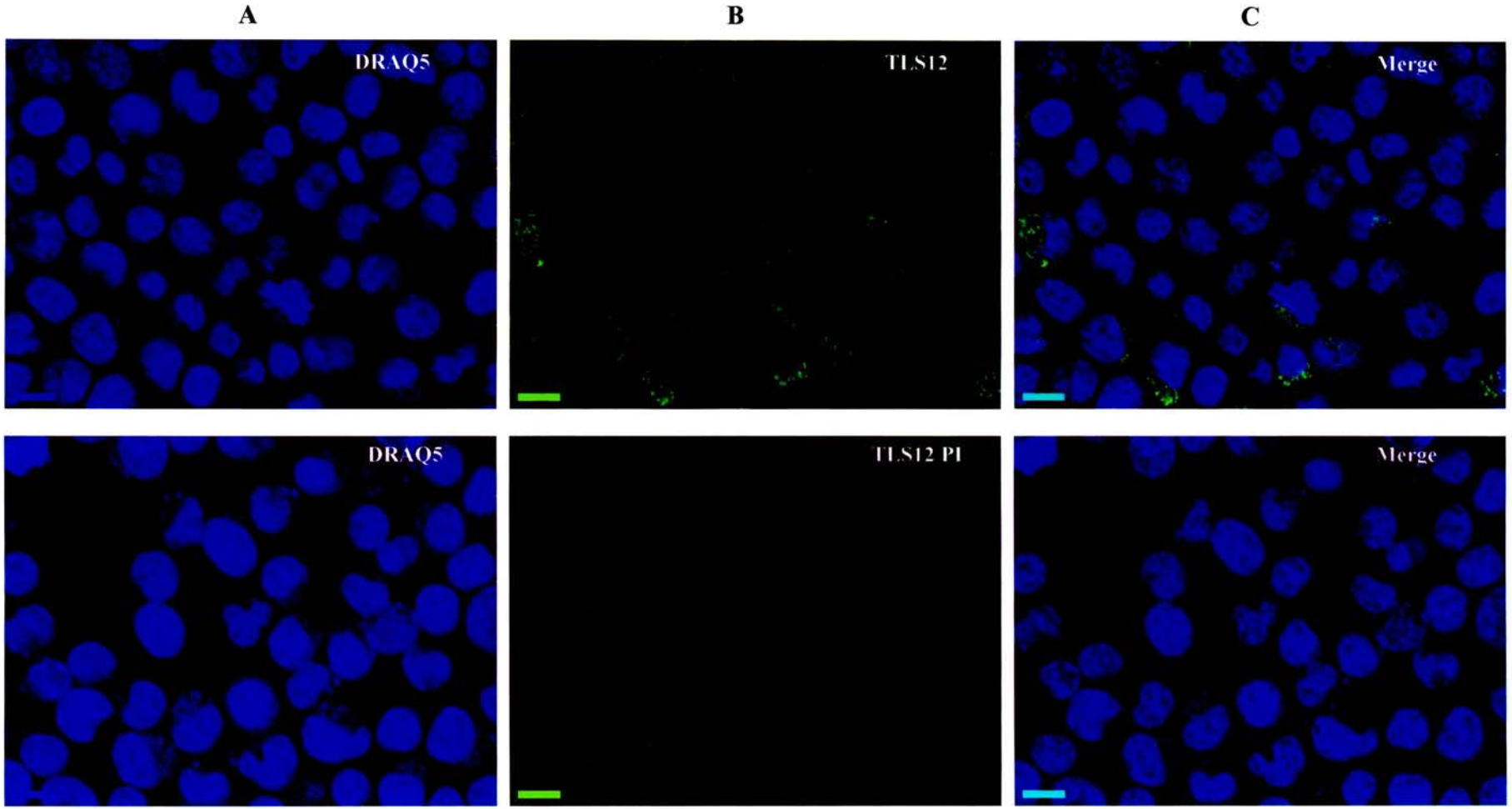


Fig. 6.4 Confocal images of TLL labelled with TLS12 serum and DRAQ5

TLL were labelled with TLS12 serum (top) or preimmune serum (bottom) at 1:150, followed by goat anti-rat FITC-IgG (4 μg/ml) and DRAQ5 diluted at 1:1000. A, DRAQ5; B, TLS12 serum/preimmune serum; C, merged images. Bars, 10 μm.

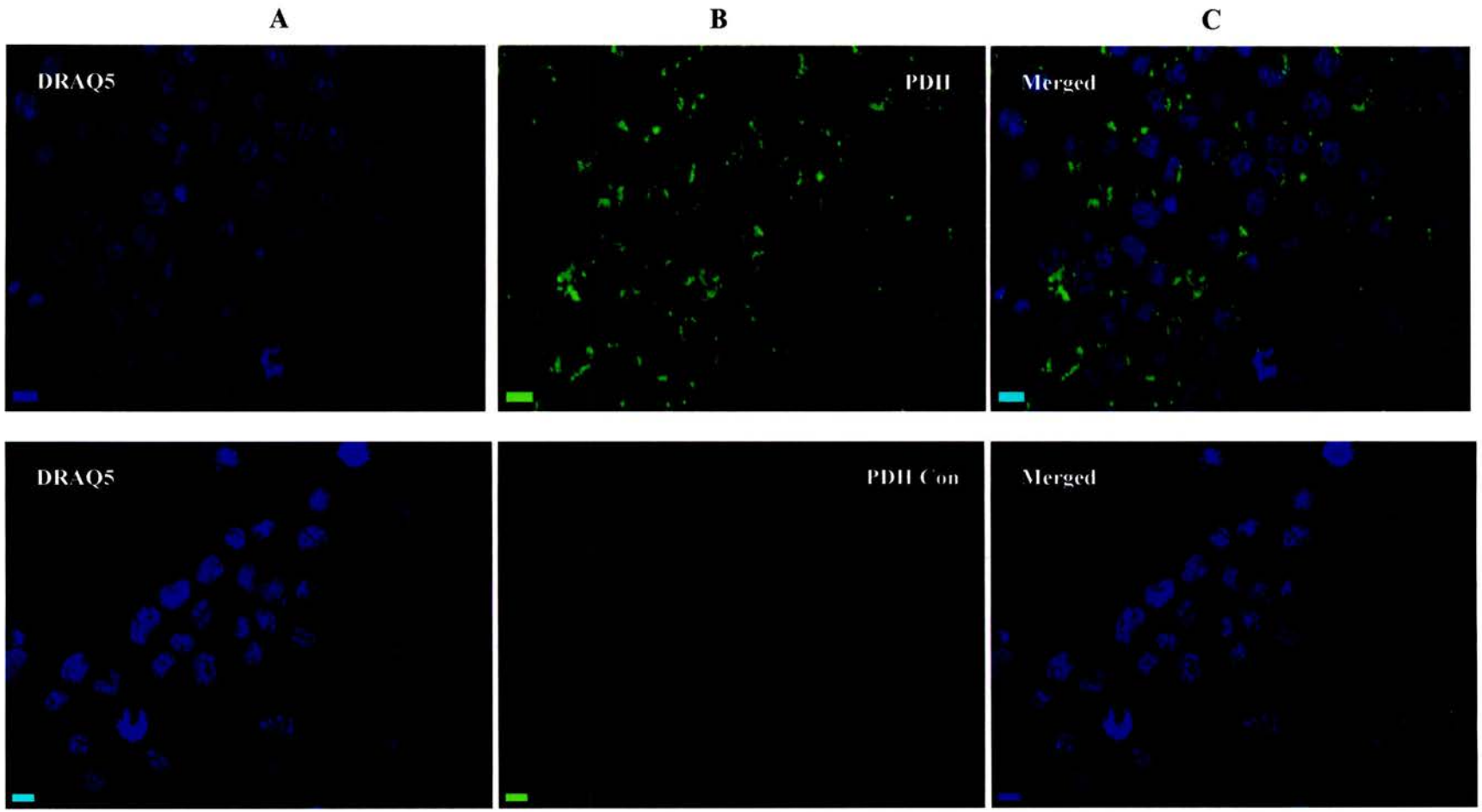


Fig. 6.5 Confocal images of TLL labelled with PDH E1β mAb and DRAQ5

TLL were labelled with PDH E1β mAb (top) or isotope matched control (bottom), followed by goat anti-rat FITC-IgG and DRAQ5. A, DRAQ5; B, PDH E1β mAb/isotope matched control; C, merged images. Bars, 10 μm.

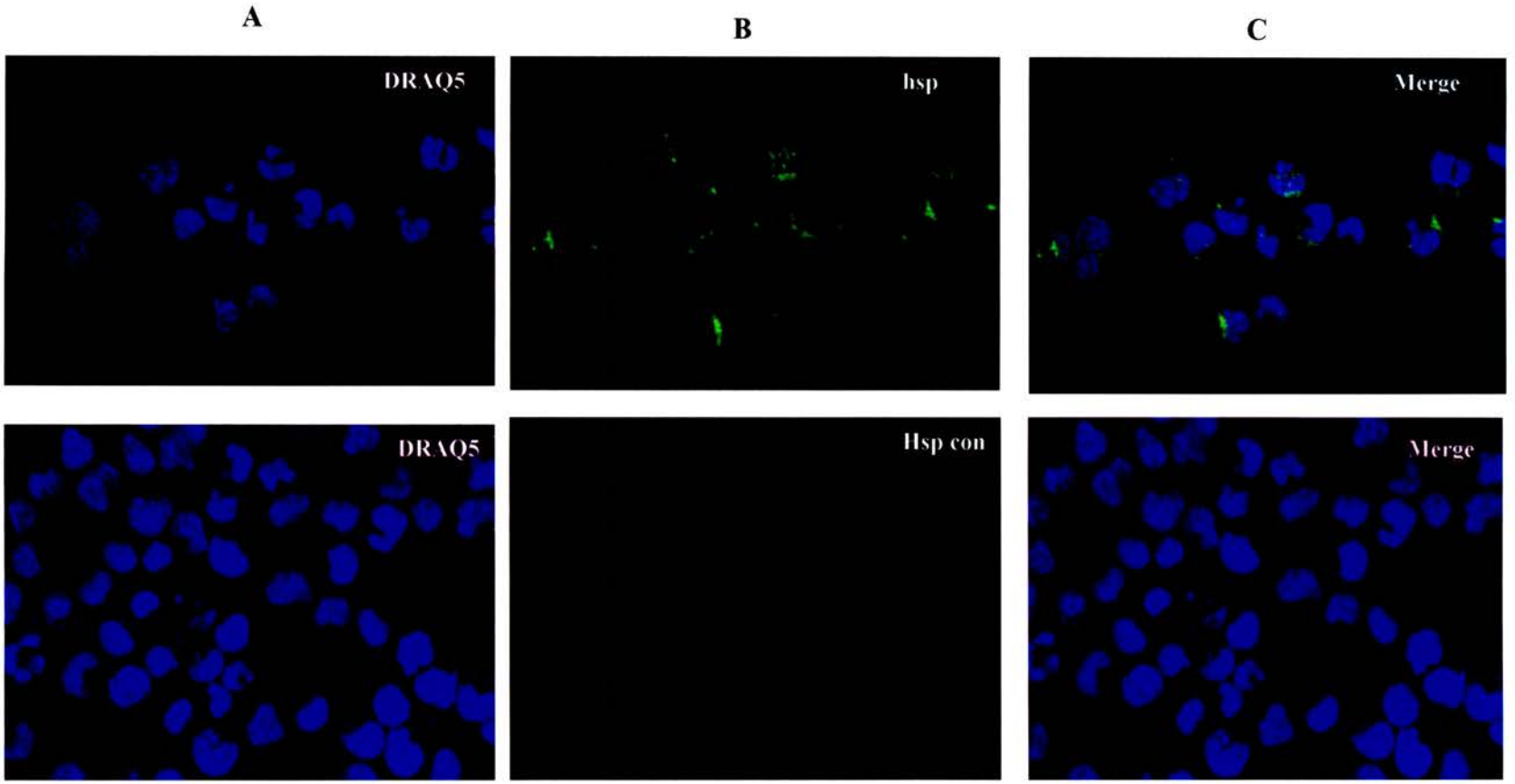


Fig. 6.6 Confocal images of TLL labelled with hsp60 mAb and DRAQ5

TLL were labelled with hsp60 mAb (top) or isotope matched control (bottom), followed by goat anti-rat FITC-IgG and DRAQ5. A, DRAQ5; B, hsp60 mAb/isotope matched control; C, merged images. Bars, 10 μm.

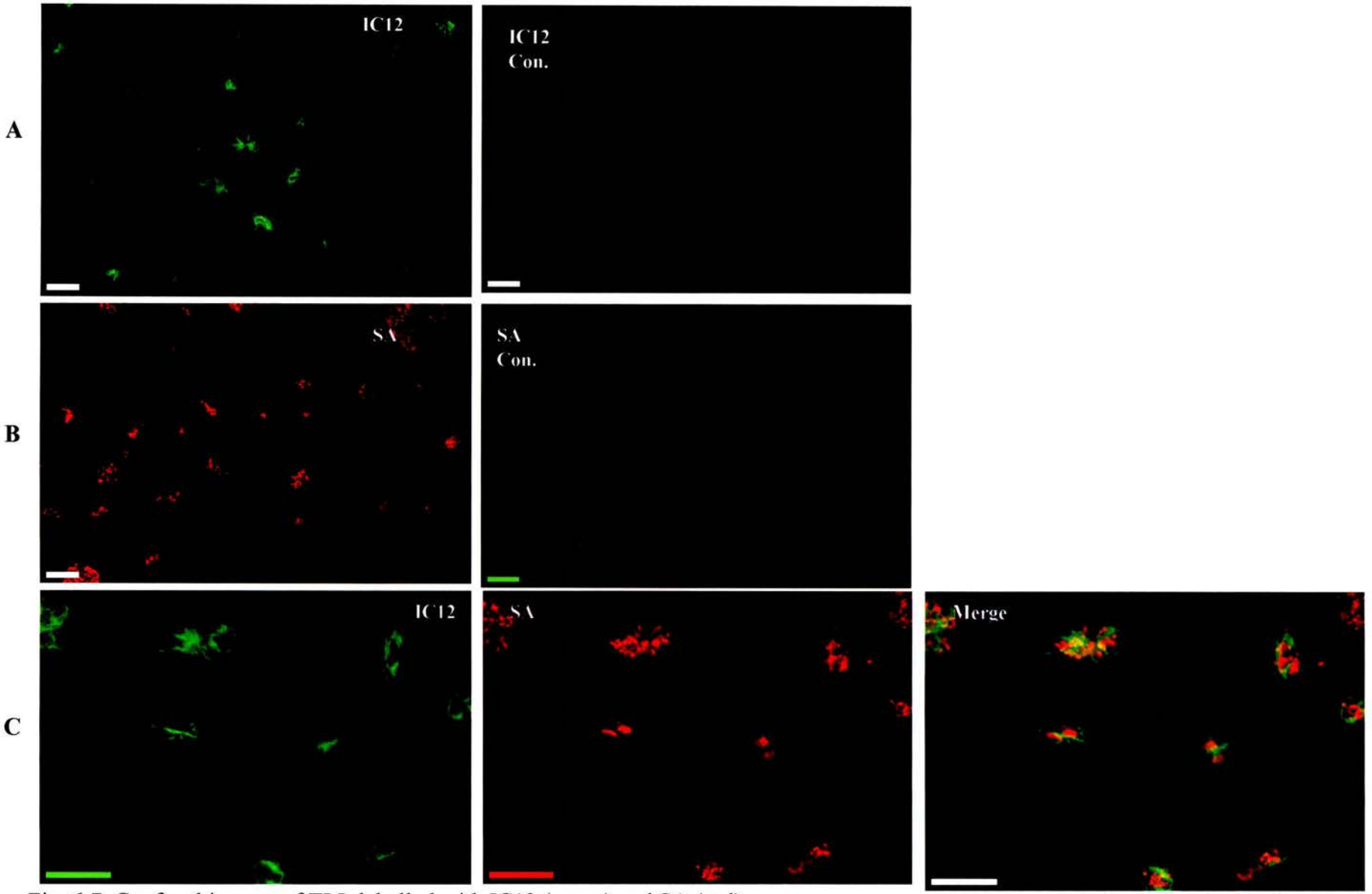


Fig. 6.7 Confocal images of TLL labelled with IC12 (green) and SA (red)

Panel A, TLL were labelled with IC12 mAb (left) or isotope matched control (right) followed by FITC-Fab of goat anti-mouse IgG. Panel B, TLL were labelled with SA serum (left) or preimmune serum control (right) followed by RRX-Fab of goat anti-rabbit IgG. Panel C, Dual-labelling of TLL with IC12 mAb and SA serum. Bars, 10 μm.

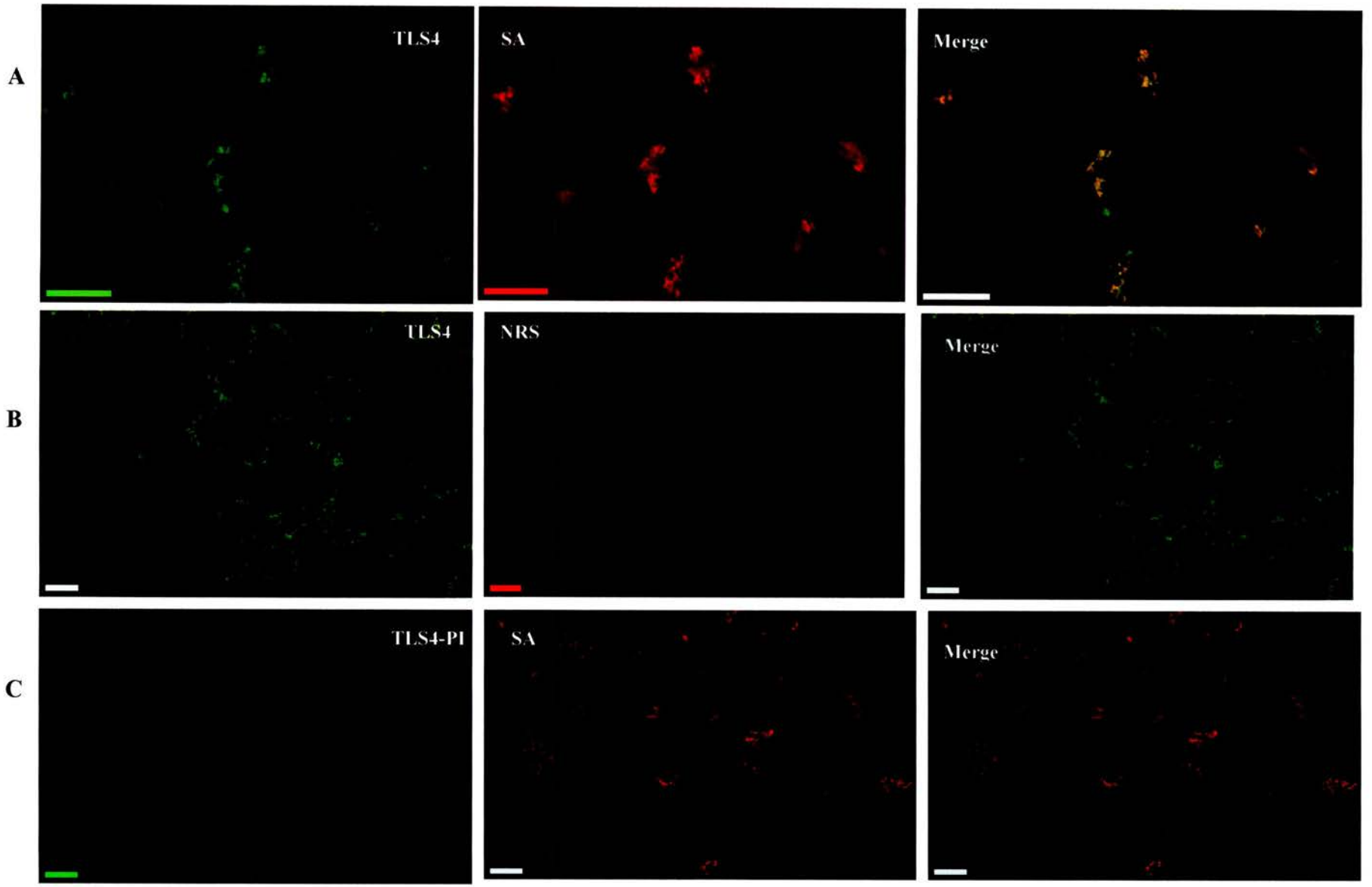


Fig. 6.8 Confocal images of TLL dual-labelled with TLS4 serum (green) and SA (red)

Panel A, Dual-labelling of TLL with TLS4 serum/FITC-Fab of goat anti-rat IgG and SA/RRX-Fab of goat anti-rabbit IgG. Panel B, Dual-labelling of TLL with TLS4 serum and SA control; Panel C, Dual-labelling of TLL with SA and TLS4 serum control. Bars, 10 μm.

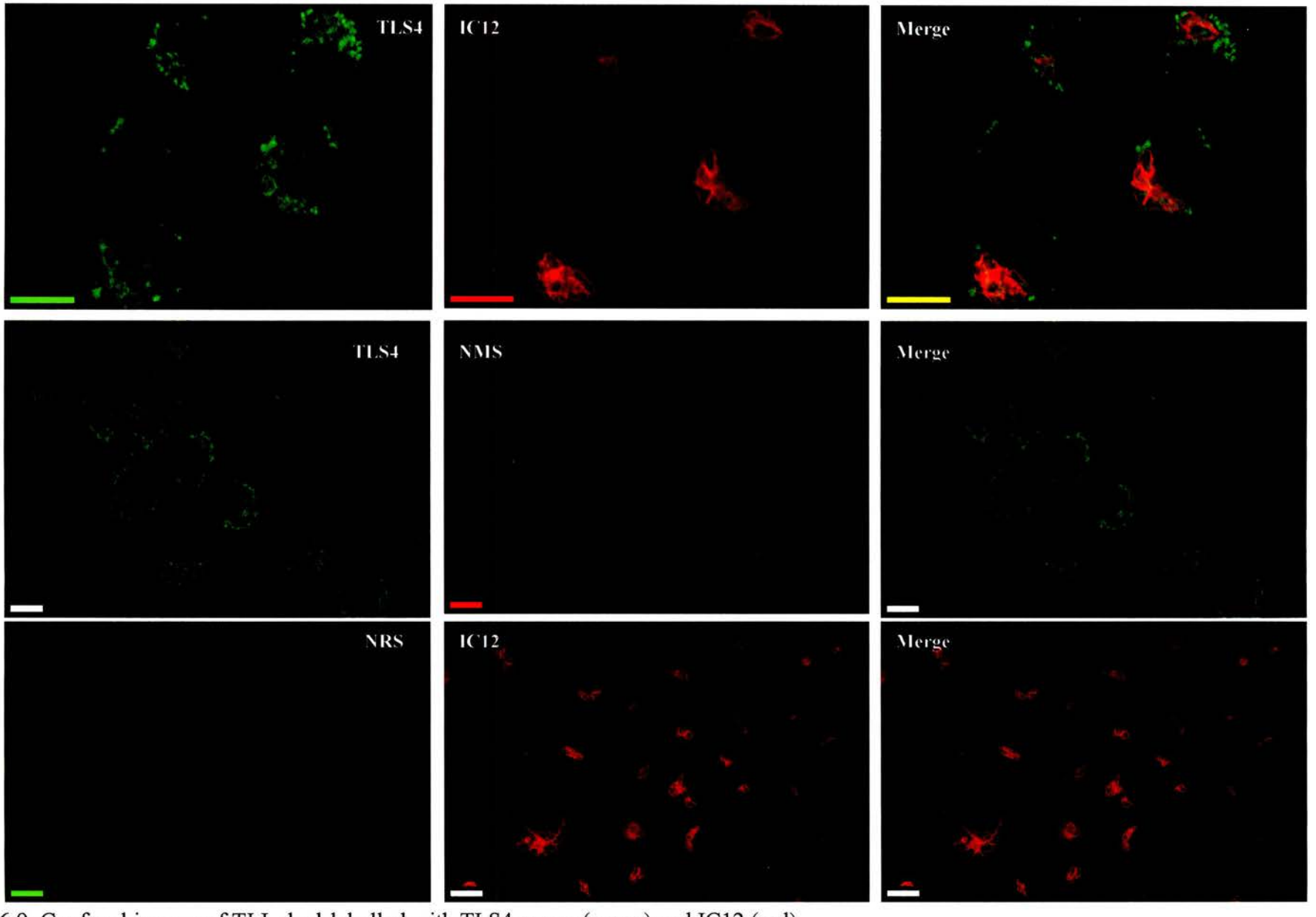


Fig. 6.9 Confocal images of TLL dual-labelled with TLS4 serum (green) and IC12 (red)

Panel A, Dual-labelling of TLL with TLS4 serum/FITC-Fab of goat anti-rat IgG and IC12/RRX-Fab of goat anti-mouse IgG. Panel B, Dual-labelling of TLL with TLS4 serum and IC12 control. Panel C, Dual-labelling of TLL with IC12 and TLS4 serum control. Bars, 10 μm.

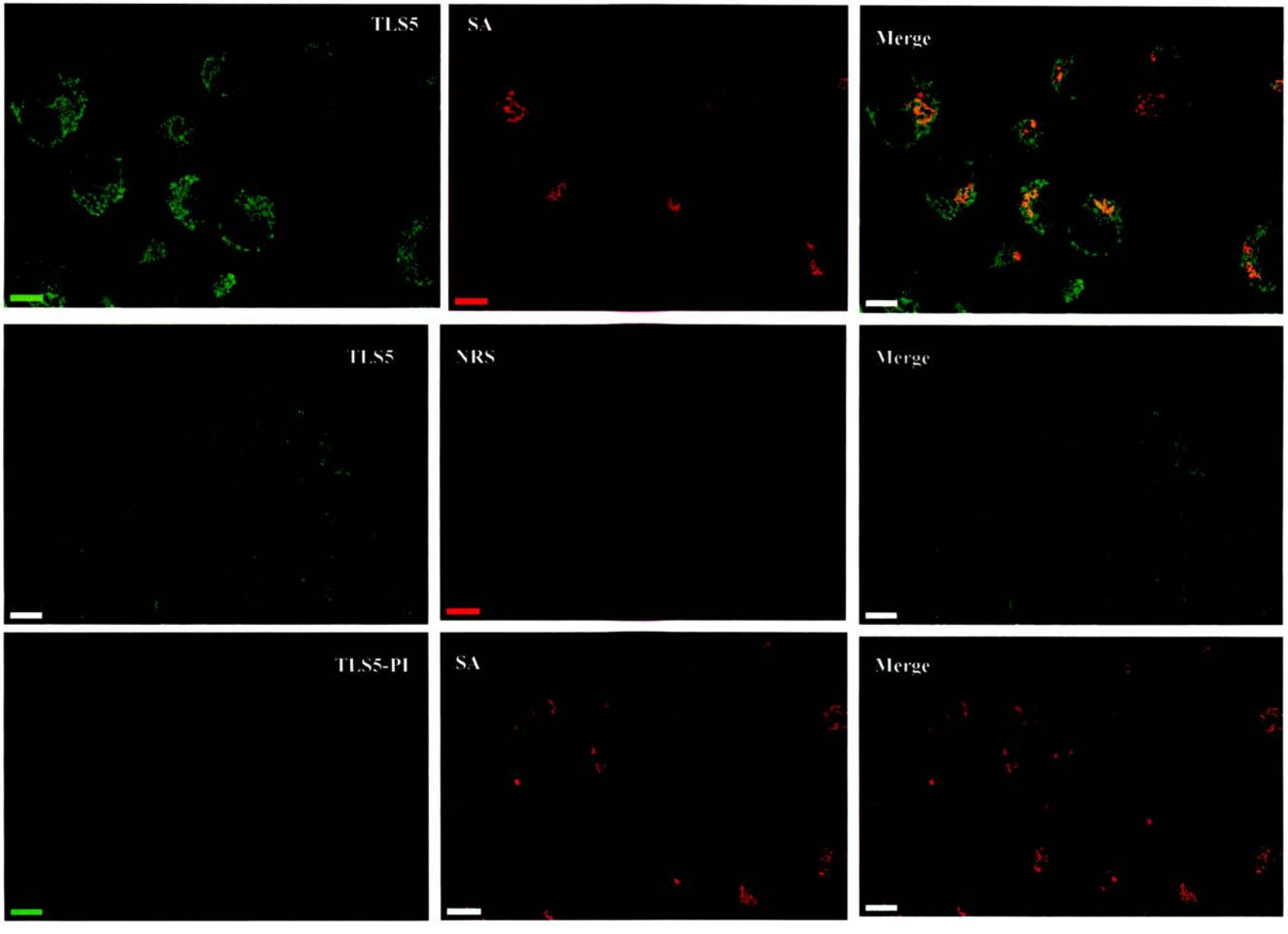


Fig. 6.10 Confocal images of TLL dual-labelled with TLS5 serum (green) and SA (red)

Panel A, Dual-labelling of TLL with TLS5 serum/FITC-Fab of goat anti-rat IgG and SA/RRX-Fab of goat anti-rabbit IgG. Panel B, Dual-labelling of TLL with TLS5 serum and SA control. Panel C, Dual-labelling of TLL with SA and TLS5 serum control. Bars, 10 μm.

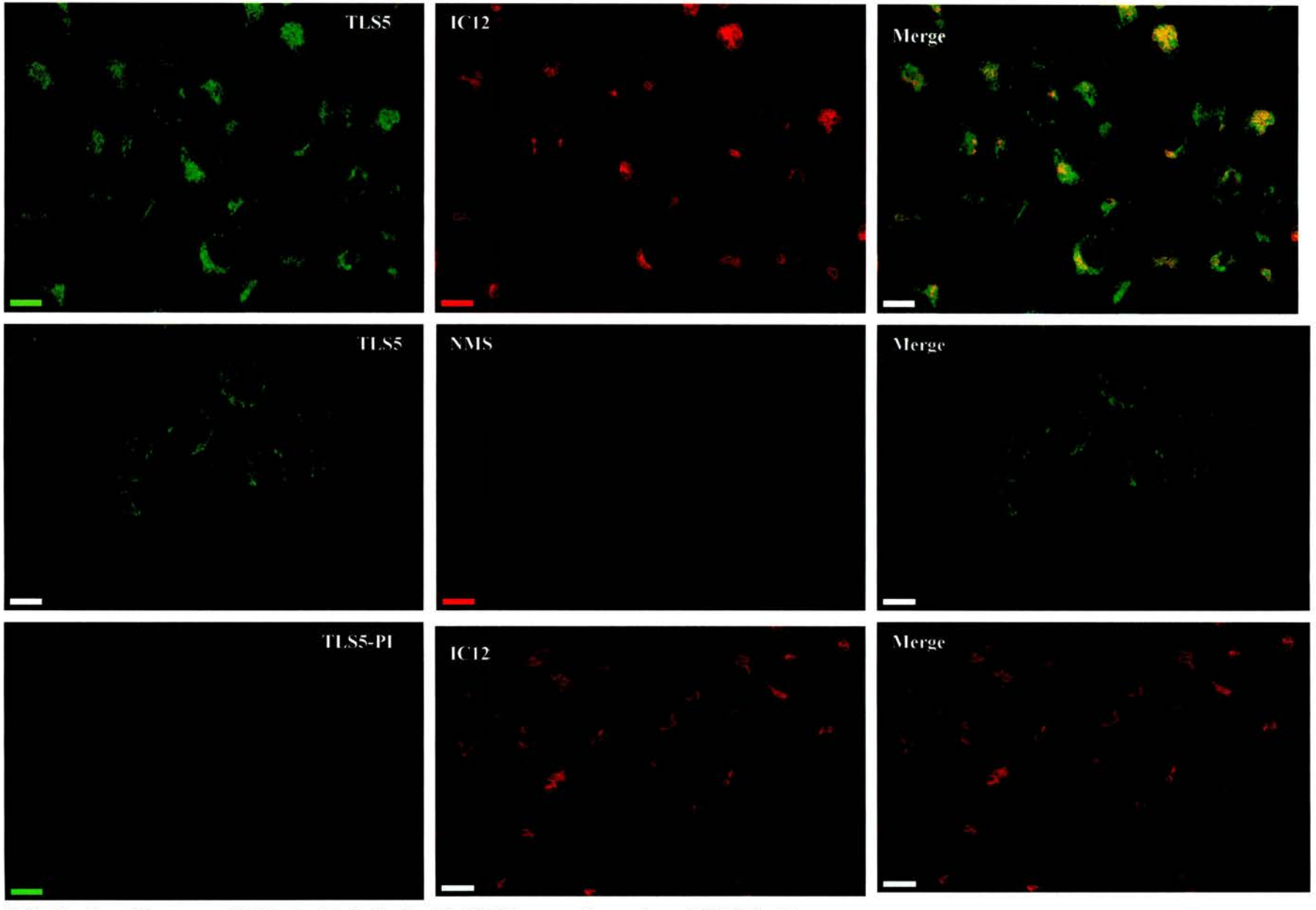


Fig. 6.11 Confocal images of TLL dual-labelled with TLS5 serum (green) and IC12 (red)

Panel A, Dual-labelling of TLL with TLS5 serum/FITC-Fab of goat anti-rat IgG and IC12/RRX-Fab of goat anti-mouse IgG. Panel B, Dual-labelling of TLL with TLS5 serum and IC12 control. Panel C, Dual-labelling of TLL with IC12 and TLS5 serum control. Bars, 10 μm.

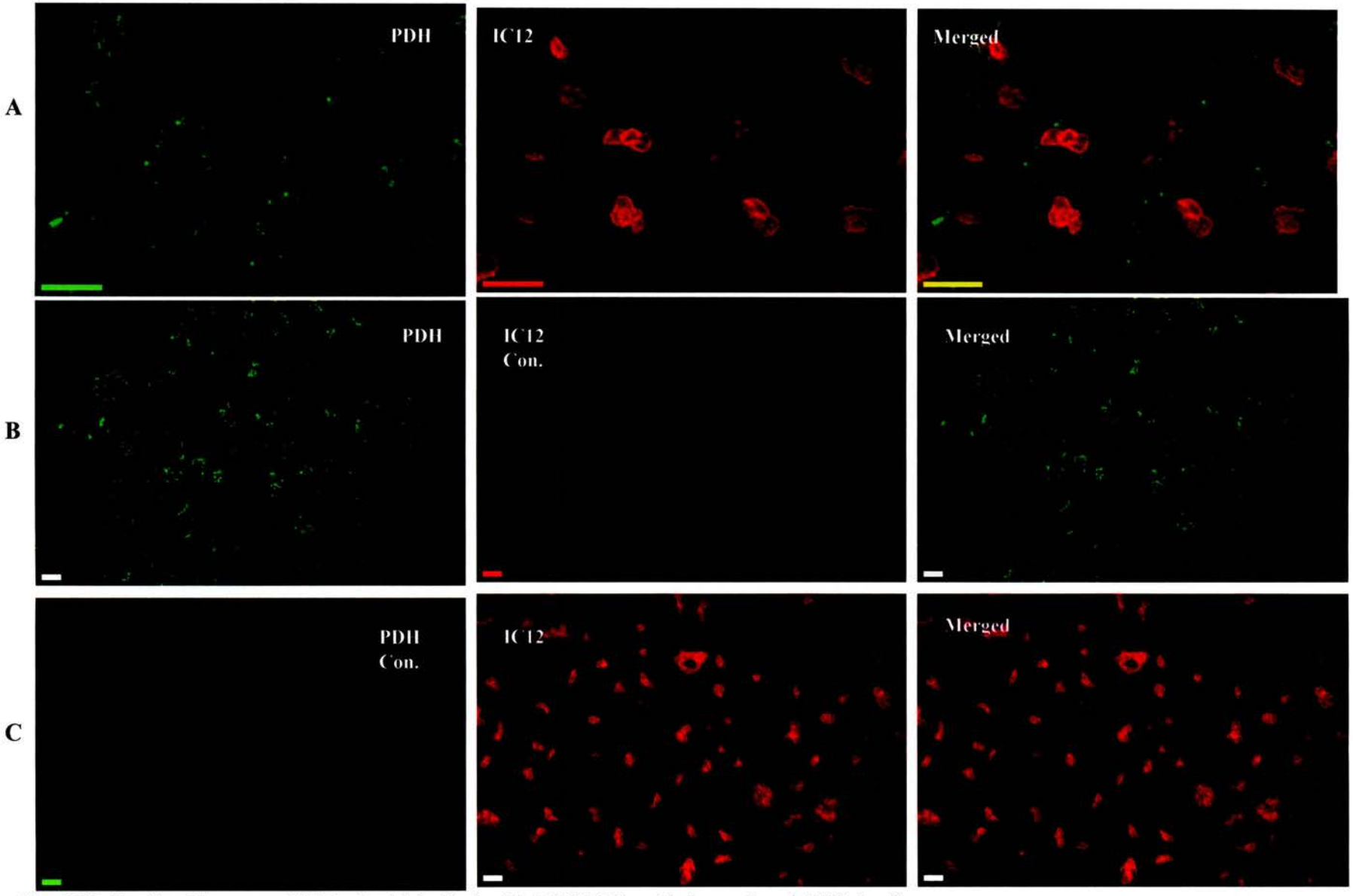


Fig. 6.12 Confocal images of TLL dual-labelled with PDH E1β mAb (green) and IC12 (red)

Panel A, Dual-labelling of TLL with PDH E1β mAb/FITC-Fab of goat anti-mouse IgG and IC12/RRX-Fab of goat anti-mouse IgG complex. Panel B, Dual-labelling of TLL with PDH E1β and IC12 isotype matched control. Panel C, Dual-labelling of TLL with IC12 and PDH E1β mAb isotype matched control. Bars, 10 μm.

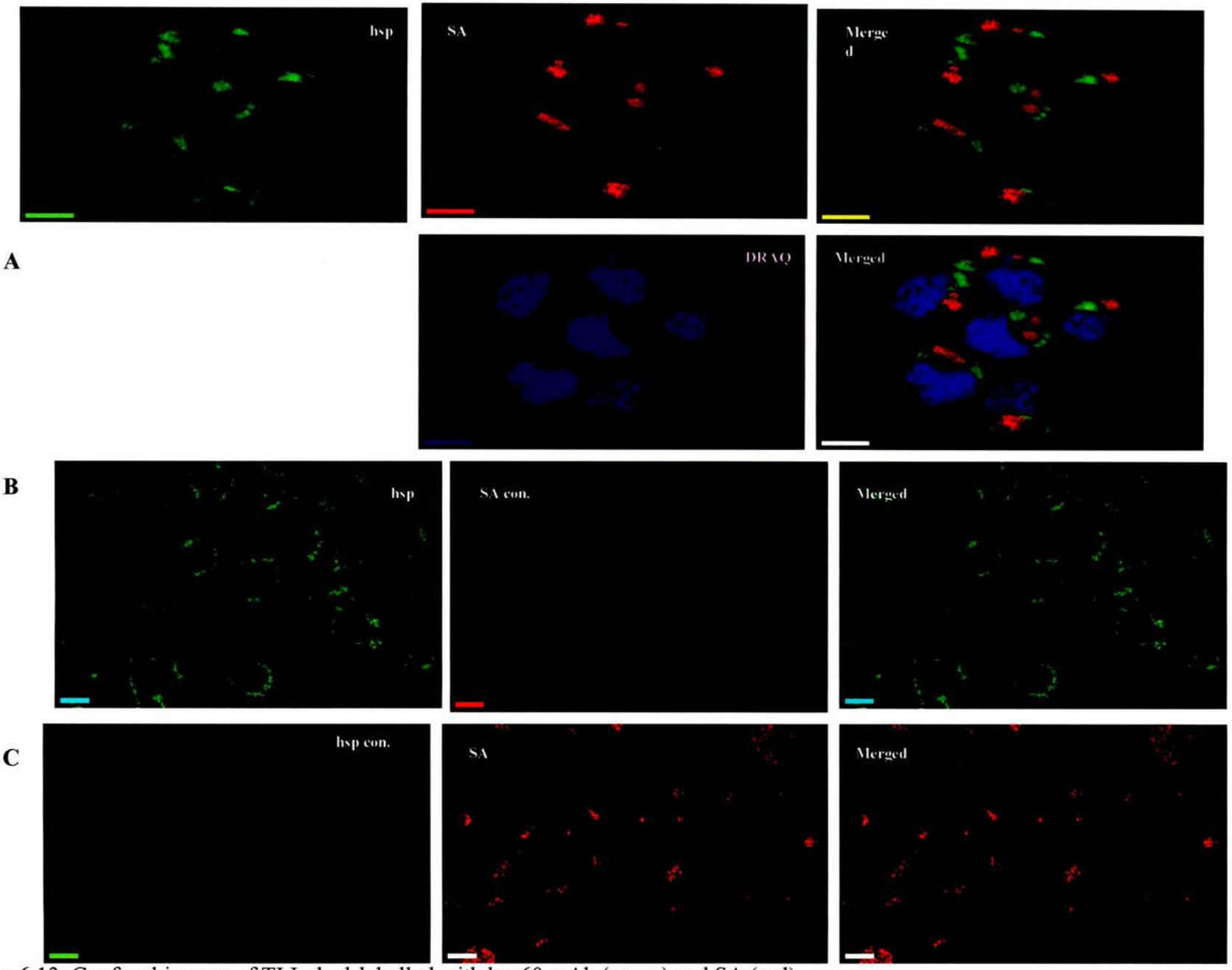


Fig. 6.13 Confocal images of TLL dual-labelled with hsp60 mAb (green) and SA (red)
Panel A,TLL were labelled with hsp60 mAb/FITC-Fab of goat anti-mouse IgG and SA/RRX-Fab of goat anti-rabbit IgG, followed by DRAQ5.
Panel B, Dual-labelling of TLL with hsp60 mAb and SA control. Panel C, Dual-labelling of TLL with SA and hsp60 mAb control. Bars, 10 μm.

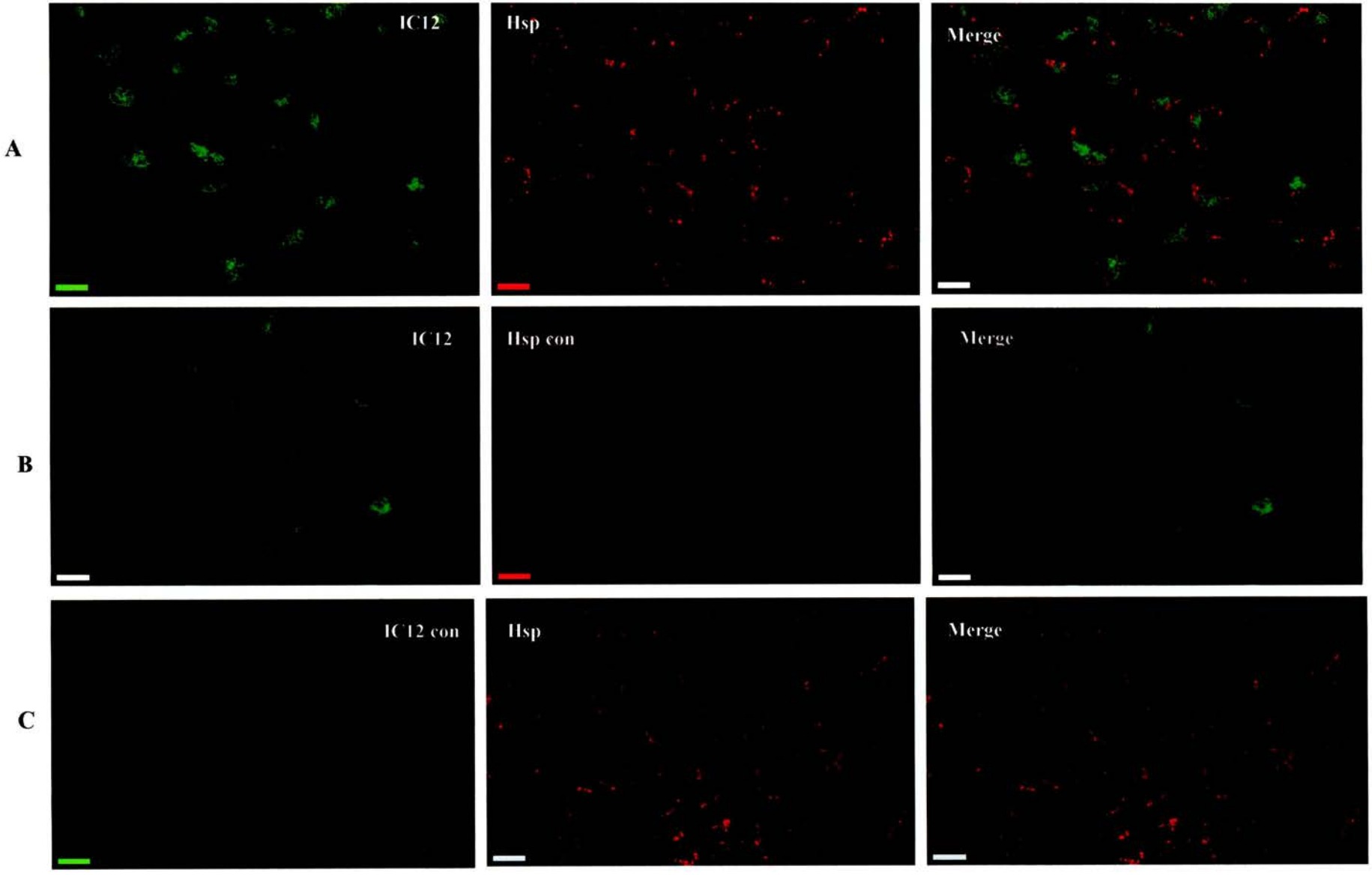


Fig. 6.14 Confocal images of TLL dual-labelled with IC12 (green) and hsp60 mAb (red)

Panel A, Dual-labelling of TLL with IC12/FITC-Fab of goat anti-mouse IgG and hsp60 mAb/RRX-Fab of goat anti-mouse IgG.

Panel B, Dual-labelling of TLL with IC12 and hsp60 mAb control. Panel C, Dual-labelling of TLL with hsp60 mAb and IC12 control.

Bars, 10 μm.

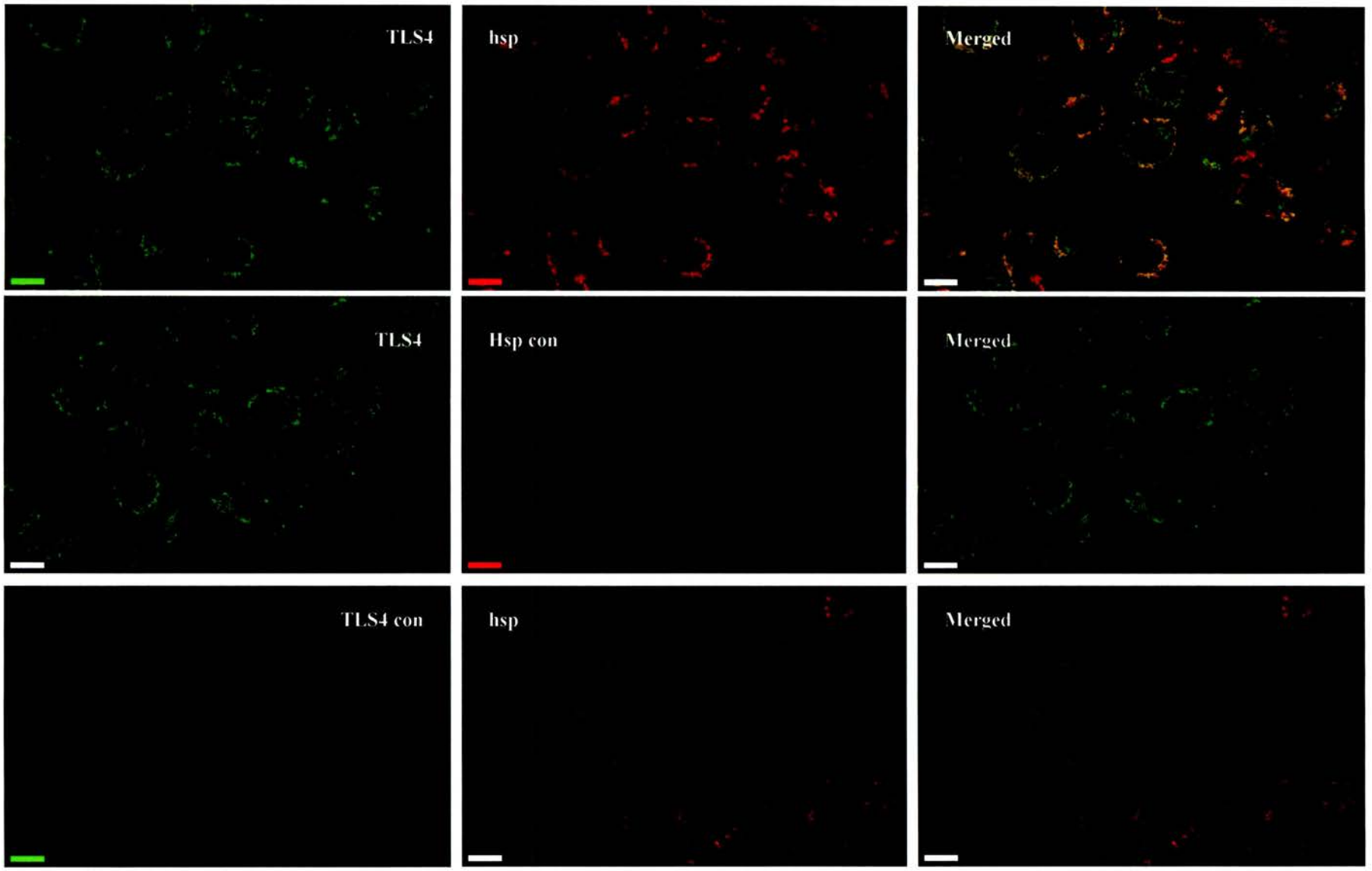


Fig. 6.15 Confocal images of TLL dual-labelled with TLS4 serum (green) and hsp60 mAb (red)

Panel A, Dual-labelling of TLL with TLS4 serum/FITC-Fab of goat anti-rat IgG and hsp60 mAb/RRX-Fab of goat anti-mouse IgG. Panel B, Dual-labelling of TLL with TLS4 serum and hsp60 mAb control. Panel C, Dual-labelling of TLL with hsp60 mAb and TLS4 serum control. Bars, 10 μm.

6.3.4 TLL and schizont analysis using MitoTracker dye

A range of concentrations of the MitoTracker dye was investigated in TLL and sheep PBMC blast cells and it was found that a final concentration of 300 nM gave optimal staining (data not shown). In normal sheep PBMC blast cells stained by MitoTracker dye, mitochondria appeared to be stained (Fig. 6.16B).The staining pattern was different in TLL, with large areas of the cell being stained rather than discrete organellar structures (Fig. 6.16C). Using the schizont specific antibody, SA, it was shown that the staining area appeared consistent with the region where schizonts were located (Fig. 6.16D), which suggested that schizonts were stained by the MitoTracker dye. Further evidence for this was obtained when the dye was used to stain schizonts isolated from TLL and the whole schizonts appeared to have taken up the dye (Fig. 6.16A).

6.3.5 TLL analysis by EM

Because MitoTracker is essentially an indicator of an oxidising environment (http://www.probes.com/servlets/product?item=22425), its failure of the MitoTracker dye to stain mitochondria within TLL raised the possibility that they had lost their membrane potential. TLL were therefore examined by EM to evaluate mitochondrial morphology. These studies revealed that many host mitochondria appeared swollen and were larger than normal size. Most of them appeared to be 1.5-2 μm in diameter with indistinct cristae (Fig. 6.17A). To investigate whether these abnormalities represented an artifact associated with sample fixation and processing, a control sample of normal PBMC blasts was processed simultaneously and found to contain mitochondria of normal morphology, with distinct cristae (Fig. 6.17B).

6.3.6 Dual-labelling of isolated schizonts

To evaluate host mitochondrial contamination in schizont preparations, isolated schizonts were dual-labelled and analysed by confocal microscopy. Dual-labelling of schizonts with SA and hsp60 mAb indicated that, while the majority of the preparation consisted of schizonts, there was also a significant presence of host mitochondria. No colocalisation of SA and hsp60 was detected (Fig. 6.18). Dual-labelling of schizonts with IC12 and hsp60 mAb showed that host mitochondria were

clustered together and some of them appeared to be associated with schizonts (Fig. 6.19). Some mitochondria also appeared to be enlarged, being approximately 2 μm in diameter. Although the dual-labelling revealed a significant host mitochondrial presence in preparations of isolated schizonts, an accurate percentage of mitochondria contamination could not be determined, because of the clustering of the organelles.

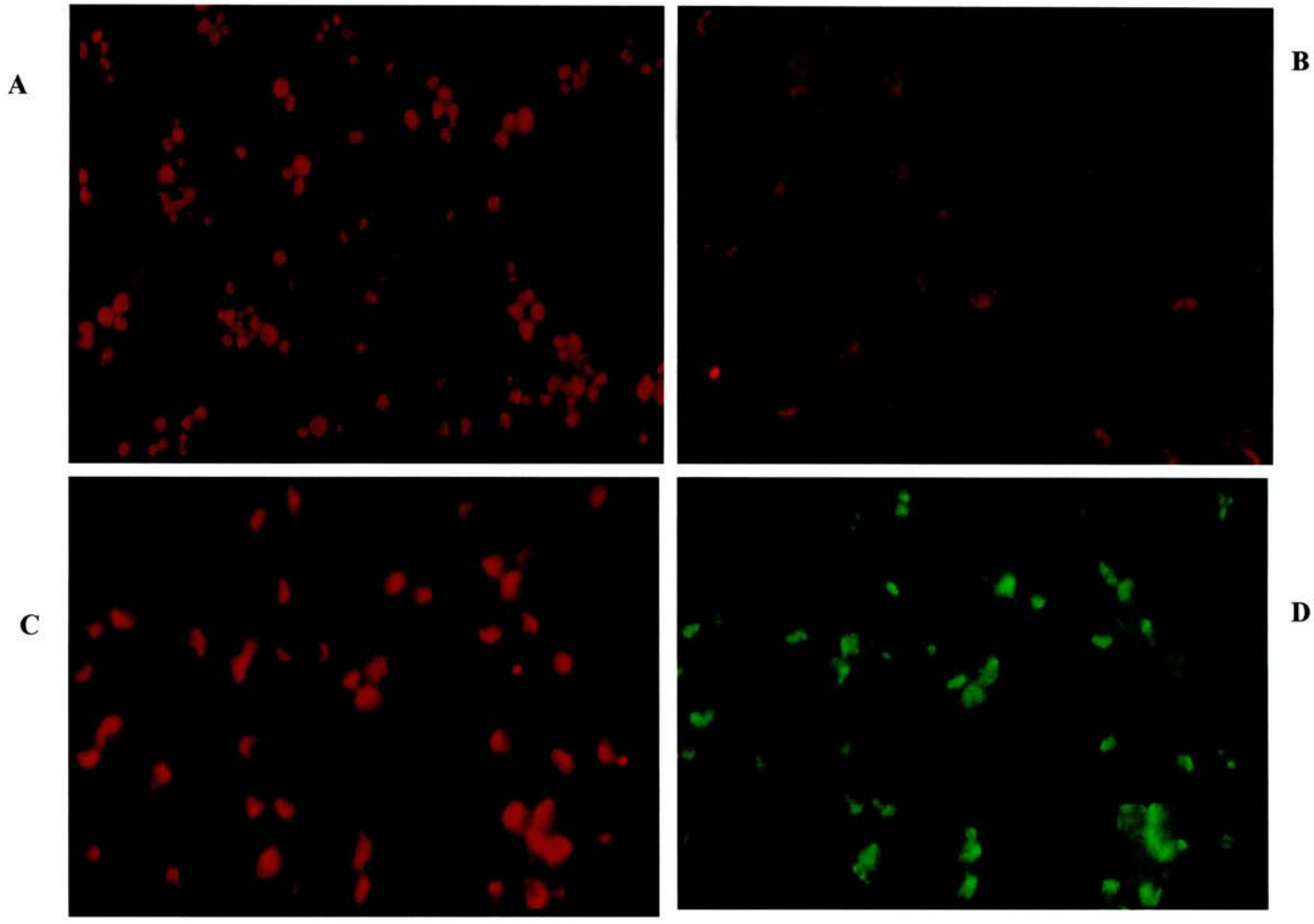


Fig. 6.16 TLL and purified schizonts analysed by MitoTracker dye staining

Purified *T. lestoquardi* schizonts (A) and sheep PBMC blasts (B) were stained with MitoTracker dye at 300 nM, × 300; TLL were stained with MitoTracker dye (C), and by labelling with *T. annulata* SA antibody (1:400) and FITC-conjugated goat anti-rabbit IgG at 1:200 dilution (D), × 400.

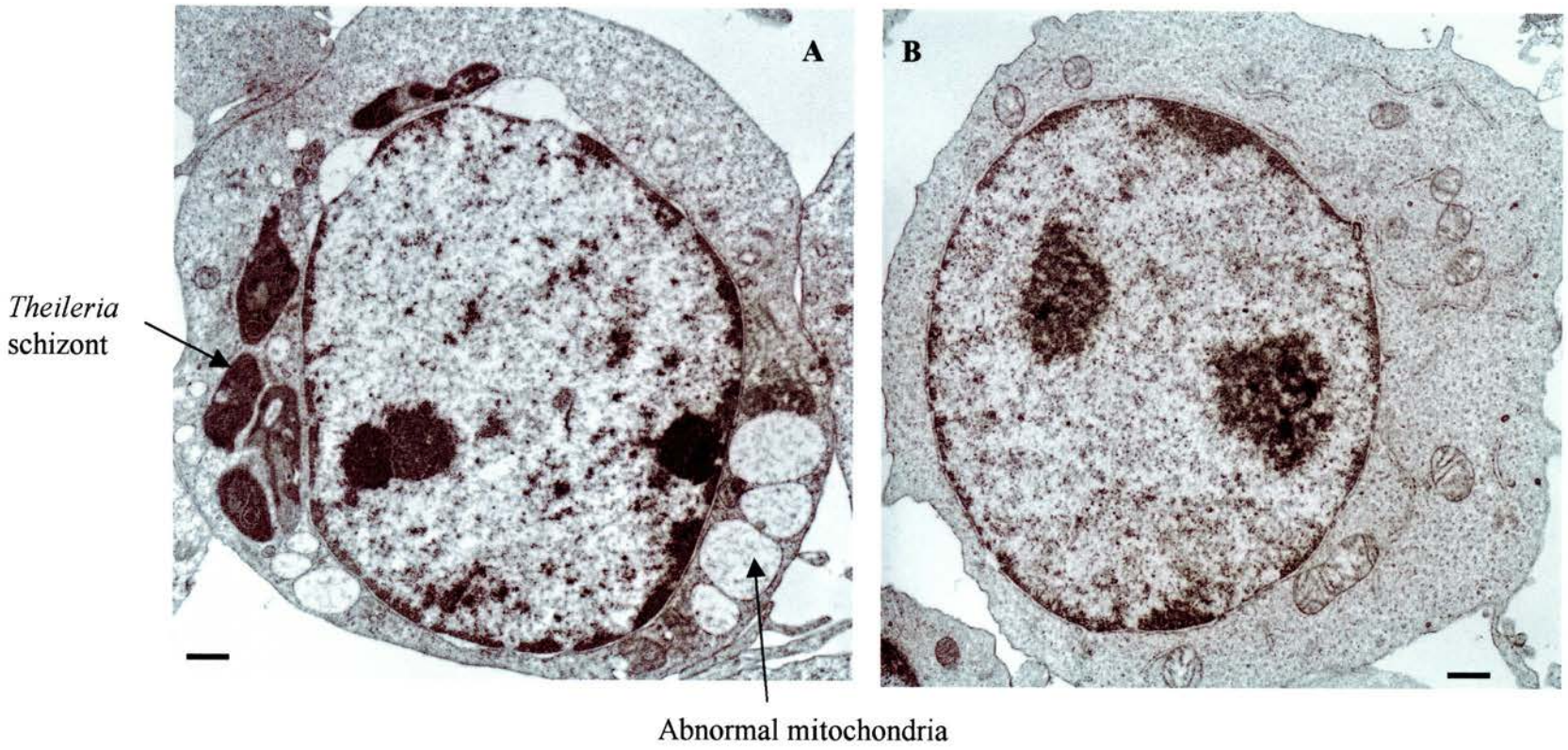


Fig. 6.17 EM analysis of TLL (A) and normal sheep PBMC blasts (B).

Abnormal mitochondria in TLL are shown by the arrow. Bar-1μm

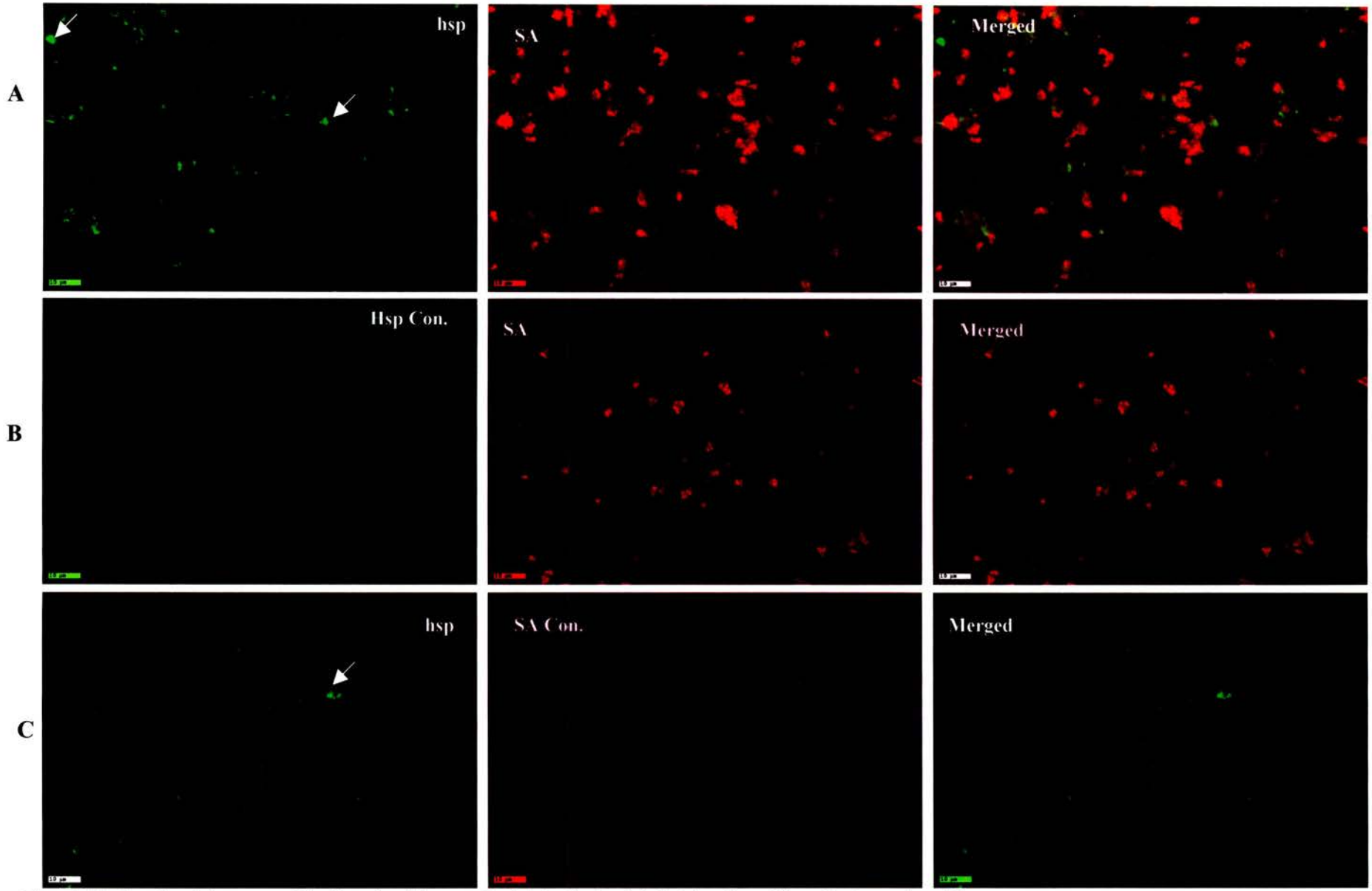


Fig. 6.18 Confocal images of isolated schizonts dual-labelled with SA (red) and hsp60 (green) mAb

Panel A, Dual-labelling of isolated schizonts with hsp60 mAb/FITC-Fab of goat anti-mouse IgG and SA/RRX-Fab of goat anti-rabbit IgG. Panel B, Dual-labelling of isolated schizonts with SA and hsp60 mAb control. Panel C, Dual-labelling of schizonts with hsp60 mAb and SA control. Some of the enlarged mitochondria are shown by arrows. Bars, 10 μm.

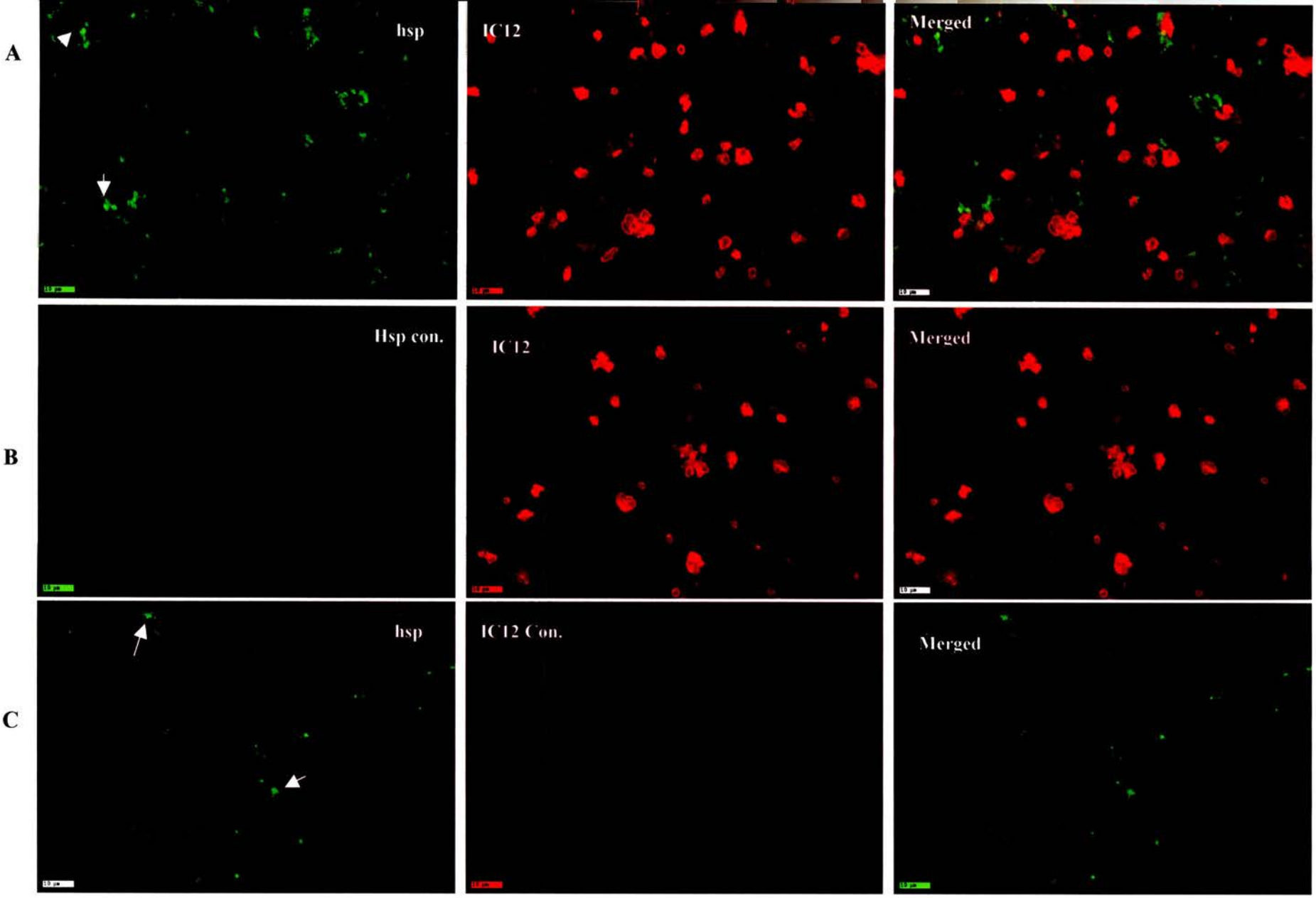


Fig. 6.19 Confocal images of isolated schizonts dual-labelled with IC12 (red) and hsp60 (green) mAbs
Panel A, Dual-labelling of isolated schizonts with hsp60 mAb/FITC-Fab of goat anti-mouse IgG and IC12 mAb/RRX-Fab of goat anti-mouse IgG. Panel B, Dual-labelling of schizonts with IC12 and hsp 60 mAb control. Panel C, Dual-labelling of schizonts with hsp60 mAb and IC12 control. Some of enlarged mitochondria are shown by arrows. Bars, 10 μm.

6.4 Discussion

To investigate the intracellular localisation of host derived proteins found to contaminate isolated schizonts, TLS4, TLS5, TLS11 and TLS12 were chosen for raising specific rat antisera for testing by IFA along with commercially available mAbs specific for hsp60 and PDH E1β. IFA analysis of rat sera raised against TLS4, TLS5, TLS11 and TLS12 lower titres of antibody (from 1: 40 to 1:150) which possibly resulted from the small amount of antigens available for their generations. Because of the difficulty associated with quantification of protein in 2DE gel spots, the exact amount could not be determined, although it was estimated that approximately 0.3-0.5 μg protein was injected per rat on each occasion. It is considered that 1-10 μg antigen is required for generating an antibody response (Harlow et al. 1988). Immuno-blot analysis with the antisera failed to reveal specific protein bands and showed high background staining (data not shown). This might simply be due to the low antibody titres.

Although no specific recognition was observed by immunoblot analysis, rat sera raised against TLS4, TLS5, TLS11 and TLS12 showed positive labelling of TLL in IFA analysis suggesting that specific antibody responses had been obtained (Fig. 6.1). However, high background was observed in preliminary immunofluorescence analysis and intracellular locations could not be determined (Fig. 6.1). To improve specific labelling and reduce background, labelling conditions were optimised using higher antibody dilutions, longer incubations and blocking with 10% normal serum from the same species as the secondary antibody (section 6.2.4). Using these conditions, single-labelling of TLL with TLS4, TLS5 and TLS12 sera showed significantly improved specific labelling (Fig. 6.2 to Fig. 6.6) compared to preliminary analysis (Fig. 6.1).

The single-labelling conditions established above provided an optimised method for dual-labelling with *Theileria*-specific antibodies. With dual-labelling, the possibility of cross-reactivity is a major consideration, especially if two primary antibodies are raised from the same or a closely related species. To address this issue, second

primary antibody and secondary Fab fragment immuno-complexes were used in the dual-labelling analysis, as described by Brown et al. (2004). Antibodies maintain their affinity for specific antigen after labelling with Fab fragments in vitro (Brown et al. 2004). Incubation of these immune-complexes with excess normal serum from the same species as the primary antibody prevents free Fab fragments from recognizing the first primary antibody in dual-labelling (Brown et al. 2004). The method was successfully employed to label samples and controls with either preimmune rat sera or isotype matched IgG showed that cross-reactivity was avoided (Fig. 6.7 to Fig. 6.15).

In addition to the possibility of cross-reactivity in the dual-labelling processes as mentioned above, there was some potential for cross-reaction between the target antigens and protein homologues in *T. lestoquardi* schizonts. This is discussed as below.

Dual-labelling of TLL with TLS4 serum and SA or IC12 antibodies showed that TLS4 was located in the host cell cytoplasm and was also associated with intracellular schizonts. In schizonts, TLS4 was observed to co-localise with SA which marks the parasite mitochondria (Fig. 6.8 and Fig. 6.9). A possible explanation for this co-localisation is that the TLS4 serum recognized TLS4 homologues of *T. lestoquardi* schizonts. Two such homologues, designated putative MRPL12 (TA06726 and TA15030), were found by searching the annotated *T. annulata* genome (http://www.genedb.org/genedb/annulata/index.jsp). However, the amino acid sequences of these homologues have only 14.7% and 17.6% identity with the host MRPL12 sequence respectively (Fig. 5.6). The chance of cross-reactivity of TLS4 serum with the putative *Theileria* TLS4 homologue therefore appears remote.

A second possibility is that the TLS4 spot also contains parasite mitochondrial proteins with similar mass and pI. The two putative parasite MRPL12 have similar predicted molecular mass and pI (TA06726, 22.7 kDa and 9.3; TA15030, 22.7 kDa and 9.4, individually) to those of the host molecule. It is therefore possible that the TLS4 spot contained these molecules. Under these circumstances, immunised rats

might be expected to have made antibodies to both host and parasite homologues, so that the sera would recognise the schizont protein. However, TLS4 always appeared as a single spot in the 2D gel (Fig. 4.7), with no prominent spots close to it. Secondly, the MALDI-TOF and Q-TOF data were consistent with only a singel protein species being present. Therefore, the chance that the rat serum was raised against two TLS4 homologues appears unlikely.

Dual-labelling of TLL with TLS4 serum and hsp60 mAb confirmed that TLS4 serum labelled mitochondria in the host cell cytoplasm (Fig. 6.15). As revealed by dual-labelling with SA, which was raised against Tamhsp70, a parasite mitochondrial antigen, the serum also stained schizont mitochondria (Fig. 6.8). This provides evidence that host MRPL12 is present within schizont mitochondria, and suggests that it may be recruited by schizonts in TLL. Mammalian MRPL12 acts as a translational regulator of mitochondrial mRNAs and dysfunction of MRPL12 activity causes reduced cell growth rate associated with the abolition of mitochondrial oxidative phosphorylation and ATP production (Marty et al. 1996; 1997; Mariottini et al. 1999). An association of host-derived TLS4 with intracellular *T. lestoquardi* schizonts might suggest that, although a putative *Theileria* L12 gene is present in the genome, it may not be functional and host derived TLS4 might be important for infected cell proliferation and parasite survival. Notwithstanding the above hypothesis, how the protein might be recruited and transported to schizonts is unclear.

Under the conditions used in this study to separate schizonts (section 2.3), mitochondria would not be expected to contaminate the preparations. However, it is possible that schizonts might be closely associated with host mitochondria thereby resulting in a significant mitochondrial presence in the schizont preparation. In *Theileria* parasites, no association of schizonts with host mitochondria has been reported although, in *T. gondii*-infected cells, a close association with host mitochondria was observed (Sinai et al. 2001). An interaction between the schizont and host microtubules has been observed in *T. parva*-infected cells (Fawcett et al. 1984; Shaw et al. 1991) and it is known that *Theileria* parasites ensure transmission

to daughter cells by associating with the spindle apparatus during mitosis (Hulliger et al. 1964). Mitochondria also associate with microtubules (Ball et al. 1982; Couchman et al. 1982) and hence the latter may act as a bridge between mitochondria and the parasite within the cell.

MitoTracker dye is a mitochondrion-selective probe and, in this study, was initially used to evaluate host mitochondrial contamination in purified schizonts. Although no evidence for mitochondrial staining was seen in schizont preparations, the parasite appeared to stain strongly. In addition, when TLL were probed with the dye, although schizonts appeared to stain, no evidence of mitochondrial staining was observed (Fig. 6.16). Although the exact mechanism of MitoTracker selective staining is unclear, it has a net positive charge and binds to the negatively charged mitochondrial inner membrane, an event which is dependent on the mitochondrial membrane potential (http://www.probes.com/servlets/product/item=22425). This mitochondria-selective dye has also been found to stain other intracellular pathogens, such as *Mycobacterium* (Penberth J. personal communication). Staining of schizonts in TLL by MitoTracker suggests that *T. lestoquardi* schizonts may have a membrane potential. However, little is known about the *Theileria* parasite plasma membrane characteristics or its membrane potential.

Similarly, the lack of demonstrable staining of host mitochondria by the MitoTracker dye (Fig. 6.16C) suggests that their membrane potential might have been lost. TLL morphology was further investigated by EM analysis. The ultrastructural morphology of mitochondria in TLL appeared abnormal, being enlarged in size and having indistinct cristae (Fig. 6.17). However, the possibility of abnormal features resulting from fixation processes must be considered. According to the description of Cheville (1994), glutaraldehyde is harsh on membranes during fixation, especially on mitochondrial membranes (Cheville 1994). In the present study, glutaraldehyde was used to fix the samples, allowing the possibility that it was responsible for the enlarged size of mitochondria and absence of clear cristae (Fig. 6.17A). However, a comparison with uninfected sheep PBMC blast cells processed simultaneously showed normal cell morphology and mitochondria of normal size and with clearly

defined cristae (Fig. 6.17B), suggesting that the abnormal features of mitochondria in TLL were not due to processing artifact. Nontheless, it is possible that the high metabolic rate of TLL may render their mitochondria more sensitive to artifacts of fixation, as has been described for highly active tissues (Cheville 1994). Although alterations in host cell nuclear morphology caused by infection with *Theileria* parasites have been reported, no apparent changes in mitochondrial morphology were observed (Schein et al. 1978; Jura et al. 1983). The possibility remains that the observation of abnormal mitochondrial morphology by EM in the present study (Fig 6.17) is associated with a loss of membrane potential that accounts for the lack of staining by MitoTracker dye (Fig. 6.16).

In summary, the dual-labelling immunofluorescence and confocal microscopy analysis provided evidence that host derived MRPL12 (TLS4) is associated with intracellular schizonts with some co-localising with schizont mitochondrial protein. The reasons for this association and how the protein is recruited to schizonts are unknown. Staining with antibodies against host derived proteins ATPase-δ (TLS5), hsp60 (TLS2) and PDH E1-β (TLS12) revealed that some host mitochondria are closely associated with the schizonts. A preliminary study using MitoTracker staining revealed that schizonts stained heavily with the dye whereas host mitochondria showed no staining. This may be associated with abnormal mitochondrial morphology observed by EM.

Chapter 7: General discussion

T. lestoquardi sporozoites invade myeloid cells, induce host cell transformation and the infected cells undergo uncontrolled proliferation (Brown et al. 1973) which causes infected animal malignant theileriosis (Hooshmand-Rad et al. 1973). In response to proliferation of *Theileria*-transformed cells, the host deploys its immune system to eliminate the pathogen using cytotoxic T lymphocytes (Morrison et al. 1998). Elucidating the precise mechanisms of transformation and the specificity of the host immune response awaits the identification of putative oncogenes and immune antigens. Schizont secreted proteins are likely to contain molecules that are potential CTL targets and are also responsible for transformation of infected cells (Nene et al. 2000). The aim of the present study was to identify putative schizont secreted proteins.

An optimised method for metabolic-labelling of schizont-infected cells with ^{35}S methionine/cysteine was therefore established. Cellular proteins of both intracellular schizonts and host cells were efficiently labelled, and radio-labelled schizonts were purified from infected cells using complement treatment followed by Nycodenz gradient separation. Schizonts remained viable in short-term culture and parasite associated proteins in culture supernatants were analysed by 2DE and autoradiography. Proteins corresponding to those present in schizont supernatants were identified in 2D gels of total schizont lysates and analysed by MS methods. MALDI- TOF analysis initially provided provisional identities for 8 putative schizont secreted proteins. Q-TOF MS and N-terminal sequencing analysis further confirmed three putative schizont secreted proteins and other host derived proteins.

The three putative schizont secreted proteins were identified as mhsp70, hsp70 and inorganic pyrophosphatase (PPase). *Theileria* hsp70 (TLS15) and mhsp70 (TLS3) were the most abundant spots appearing in 2DE gels (chapter 4) and their intracellular locations have been studied previously.

T. annulata mhsp70 (Tamhsp70) has been identified and found constitutively transcribed throughout the life cycle of the parasite (Schnittger et al. 2000).

Immunofluorescent staining and immunoelectron microscopy indicated that Tamhsp70 resides predominantly within schizont mitochondria and also in the host cell cytoplasm, suggesting it is secreted by schizonts (Schnittger et al. 2000a). In the present study (chapter 3), *Theileria* mhsp70 was identified in *T. lestoquardi* schizont culture supernatant by Western-blotting using a rabbit Tamhsp70 antiserum, which implies that schizonts transport the mhsp70 to the culture supernatant. This provides evidence that the Tamhsp70 homologue can be secreted by schizonts into the cytosol of infected cells.

Theileria hsp70 also belongs to the hsp70 family (Mason et al. 1989) and it has been suggested that it might play an important role in parasite-host interactions (Young 1990). The *T. parva* hsp70 gene (Tphsp70) has been found to be expressed constitutively in sporozoites, schizonts and piroplasms (Daubenberger et al. 1997). A protein homologue of Tphsp70 was identified in *T. lestoquardi* schizont culture supernatant (chapter 4), which suggests that it is secreted by schizonts. It was not observed in the host cell cytoplasm in a study by Daubenberger et al. (1997), perhaps because Tphsp70 was present in the host cytoplasm at levels too low to be detected by the method used. In *P. falciparum* infected hepatocytes, a monoclonal antibody recognizing a C-terminal fragment of the *P. falciparum* hsp70 (Pfhsp70) was used to show that Pfhsp70 was expressed on the infected hepatocyte membrane (Renia et al. 1990), which suggests that Pfhsp70 is secreted by the parasite into the host cytoplasm and, in some way, is transported to and presented on the host cell membrane.

In addition to being secreted by different parasite species, hsp70 and mhsp70 may be involved in antigen processing and presentation for CTL responses (Udono et al. 2001). Although the role of hsp in immunity to pathogen infection is poorly understood, especially in the way in which pathogen derived hsps are processed and presented on the host cell surface, hsp70 and mhsp70 appear to serve as important antigens in protective responses against infectious intracellular pathogens and induce strong humoral and cellular immune responses (Kaufmann et al. 1991). *Theileria* mhsp70 was found in host cytoplasm and has peptide motifs with potentially very high

binding affinity to bovine class I A20, and it has been suggested that antigen may be a potential antigen target for presentation by MHC I in a host CTL immune response (Schnittger et al. 2000).

Studies have shown that hsps, including members of the hsp70 family, are immune targets of γδ T cells (Himeno et al. 1996; Kimura et al. 1996). γδ cells are a subset of T cells that express a TCR consisting of a γ-chain and a δ-chain (Kaufmann 1996). They are considered to be a link between the innate and adaptive immune systems and can in some cases recognize antigen without involvement of MHC class I or class II molecules (Hayday 2000; Tanaka et al. 1995). The association of γδ T cells with many different infections, particularly those caused by intracellular pathogens, has led to the suggestion that γδ T cells constitute an innate surveillance system that acts as a first line of defence against infectious diseases (Kaufmann, 1996).

γδ T cells reactive with bacterial hsp70 are specifically activated in experimental listeriosis of mice and have been associated with protection (Kimura et al. 1996; Kimura et al. 1998). Depletion of γδ T cells with monoclonal antibodies has been shown to increase listerial multiplication (Hiromatsu et al. 1992). Reactivity of γδ T cells with bacterial hsps has also been demonstrated in humans, with vigorous responses to recombinant mycobacterial hsps being observed (Haregewoin et al. 1989). Hsp65 expression on macrophages was increased dramatically in *T. gondii* infected mice and its expression was closely correlated with increased numbers of γδ T cells and protection against the infection. It was found that depletion of γδ T cells in vivo, by administering monoclonal antibody specific to the γδ-TCR, reduced the resistance to *T. gondii* infection (Himeno et al. 1996).

In addition to the CD8+ T cell immune response described previously, it has also been suggested that γδ T cells play a role in preventing and eliminating *Theileria* infection. Daubenberger et al. (1999) reported that bovine γδ T cells are activated by *T. parva*-infected cells and lyse infected cells by recognizing conserved parasite-induced or parasite-derived antigens in an MHC-unrestricted fashion. Following infection with

T. parva sporozoites, the proportion of γδ T cells with this specificity in the animal increases 20 fold by 7 days (Morrison et al. 1995b). Collins et al. (1996) reported that freshly isolated WC1+ γδ T cells derived from *T. annulata*-infected cattle proliferated in response to parasitised cells. They also demonstrated that bovine γδ T cells expand in naïve animals during a primary infection and in immune cattle during challenge (Collins et al. 1996).

Although γδ T cell responses have been found in *T. parva* and *T. annulata* infections, no direct information is available on their antigenic specificity. However, Daubenberger et al. (1999) observed that γδ T cells reactive with *T. parva*-infected cells also recognised heat-stressed but not untreated ConA treated blast cells. In addition, recognition was not MHC-restricted. Although not conclusive, these observations are consistent with the possibility that hsps are involved in *Theileria*-specific γδ T cell responses. Since hsp70 and mhsp70 have been found as immune targets for γδ T cells in other pathogens, the identification of *Theileria* mhsp70 (TLS3) and hsp70 (TLS15) as the most dominant spots in 2D gels (chapter 3) raises the possibility that these proteins may be potential targets for γδ T cells in *T. lestoquardi* infection.

A putative inorganic pyrophosphatase (PPase) was identified in the *T. annulata* genome by Q-TOF analysis of the TLS16 spot (chapter 5). The potential function is unknown in *Theileria* parasites, but information can be obtained from homologous proteins in other species.

PPases are widely distributed among living cells and catalyze the hydrolysis of inorganic pyrophosphate (PPi) into inorganic ortho-phosphate (Pi). High-energy phosphoanhydride bonds of pyrophosphate can serve as an important alternative energy source:

$$PPi + H_2O \xrightarrow{PPase} 2Pi + 33.4\ KJ/mol$$

PPases play an important role in energy metabolism, providing a thermodynamic pull for many biosynthetic reactions (Kornberg et al. 1999). There are two main categories

of PPases, the soluble PPases and membrane-associated H+-translocating PPases (H+ PPases). H+ PPases are the primary H+ pumps and use PPi instead of ATP as an energy source (Zhen et al. 1997). In the protozoan parasites, *P. falciparum*, *L. donovani* and *T. brucei*, they have been identified and shown to translocate protons across membranes using the potential energy released by the hydrolysis of the phosphoanhydride bond of PPi (McIntosh et al. 2002).

The *Theileria* enzyme is a soluble PPase and very few studies of soluble PPases have been performed in parasites. A PPase was identified in *Ascaris suum*, a helminth parasite with zoonotic significance (Islam et al. 2003). The *A. suum* PPase (asPPase) has 40 % amino acid identity with *Theileria* PPase (Fig. 5.10). The enzyme was found in excretory/secretory (ES) products from a variety of parasite development stages, including embryonated eggs, L3, lung-stage L3 and adult male and female worms. This indicates that asPPase is expressed and secreted at all stages of the parasite's growth and it has been suggested that it has a crucial role in the process of parasite development and moulting (Islam et al. 2003). Although the *Theileria* PPase has not been characterised, it does not appear to have a signal sequence. Nonetheless, the identification of PPase in culture supernatant suggests that it may be secreted by schizonts. Given the rapid rate of proliferation of transformed cells, supply of ATP may be limiting. Under these circumstances, PPi may serve as an alternative source of energy for the parasite.

Most (70%) of the proteins identified in the study were of host origin and all originated in the host mitochondrion. While this may have some biological significance, it is most likely to have arisen from contamination of schizont preparations with host mitochondria (chapter 6).

Several studies have described purification of *Theileria* schizonts with high level of purity and yield (Sugimoto et al. 1988; Goddeeris et al. 1991; Baumgartner et al. 1999). Contamination of isolated schizonts by host nuclei and membranes was evaluated by Western-blotting and northern-blotting analysis in these studies. However, they did not investigate whether preparations were contaminated by

mitochondria (Goddeeris et al. 1991; Baumgartner et al. 1999), since schizonts were presumably regarded as being distinct from mitochondria. Furthermore, no analysis of purified schizonts was carried out at the protein level. Given the observation of the present study, it is likely that schizont samples obtained from the above methods contained a number of host derived proteins.

Generally, centrifugation at 16, 000 × g for 15 min (Lodish et al. 1999) or 13, 000 × g for 1 hr (Sinai et al. 1997) is required to pellet mitochondria of normal size and density and lower centrifugation forces would not be expected to pellet them efficiently. According to the schizont purification method described in chapter 2 (section 2.2), TLL lysates were fractionated on Nycodenz gradient media after centrifugation at 3, 360 × g for 30 min and a thick band containing the schizonts was collected from the interface. Normal mitochondria would not be expected in the band under these conditions. After collection of the schizont enriched band, the schizonts were washed 3 times by centrifugation at 1900 × g 15 for min. Even with some host mitochondrial contamination collected from the interface, it would be expected to be minimal after washing. The major concern with schizont purification was that they would be contaminated with host cell nuclei, because it was found previously that they have similar density to schizonts (Goddeeris et al. 1991). Results in the present study (chapter 2) revealed that isolated schizonts were free from host nuclei. However, subsequent investigation of schizont preparations, using mitochondria-specific antibodies, revealed significant mitochondrial contamination (chapter 6), which suggests that the schizonts co-purified with host mitochondria. Co-purification with schizonts implies either that the mitochondria have a similar density and size to schizonts and cannot be separated from them even after centrifugation 1900 × g 15 min 3 times, or, that the host mitochondria are closely associated with schizonts.

No morphological changes have been reported in mitochondria of *Theileria*-infected cells previously. However, it has been observed that, in *T. gondii*-infected cells, some host mitochondria are enlarged, with an absence of clear cristae (Sinai A. personal communication). In microsporidia infected cells, the host cell mitochondria also exhibited abnormal morphology. Some elongated mitochondria were observed to

stretch for approximately half the length of the host cell (Scanlon et al. 2004) which suggests that microsporidial infection may result in morphological alterations in some host mitochondria. EM analysis of *T. cruzi* infected myocytes has also shown aberrant morphology of some mitochondria, with loss of structure and function, including repression of transcripts for various components of the Oxphos pathway. Mitochondria isolated from infected mice exhibited substantial decline in complex IV activity. With progression of the disease, transcripts encoding polypeptides of Oxphos complexes, specifically complex I and IV, were dramatically decreased in the myocardium of infected mice when compared to normal murine hearts (Garg et al. 2003).

EM analysis of TLL as described in chapter 6 indicated that some host mitochondria appeared to have abnormal features, including an enlarged size and a loss of cristae. The mechanism behind this is unclear and it may relate to artifact of fixation. However, it raises the possibility that altered mitochondria may have a similar size and density to *T. lestoquardi* schizonts and therefore co-purify with them under the conditions used in the present study.

A perhaps more likely explanation for the co-purification of mitochondria with schizonts is that the two are closely associated within the infected cell. In this respect, it is relevant that a close association of schizonts with an orderly array of host cell microtubules has been observed (Hulliger et al. 1964; Fawcett et al. 1984). It was proposed earlier that microtubules assist in the division of schizonts during mitosis (Hulliger et al. 1964). Examination by EM showed that there are regular bridges between microtubules and the schizont surface (Fawcett et al. 1984), implying a direct linkage between the microtubules and parasite surface molecules. However, whether these schizont associated microtubules are themselves associated with other host cell organelles, such as mitochondria, has not been determined.

Microscopic analysis has revealed colocalisation of mitochondria and microtubules in diverse cell types, including mammalian cultured fibroblasts (Couchman et al. 1982) and fission yeast (Yaffe et al. 1996). Association of mitochondria with

microtubules is further supported by the observation that mitochondria redistribute in cultured mammalian cells treated with agents that disassemble microtubule networks (Ball et al. 1982). Furthermore, disruption of microtubules by certain conditional mutations in genes encoding microtubule subunits caused aberrant distribution of mitochondria in *Schizosaccharomyces pombe,* providing genetic evidence that microtubules govern mitochondrial distribution (Yaffe et al. 1996).

An investigation of the intracellular microsporidian parasite (*Nosema* sp.) revealed that, in both interphase and metaphase cells, host cell mitochondria were directly attached to the parasitophorous vacuole (PV) in which parasites reside, and that the association between the two was extremely close (Canning et al. 1997; Scanlon et al. 2004). Further, EM observations revealed that both PV and their associated host cell mitochondria were exclusively localized in the region of host cell microtubules (Terry et al. 1999). *T. gondii* parasites also reside in a specialized PV membrane (PVM) following infection of host cells, and intimate and extensive interactions between the PVM and host cell mitochondria have been observed (de Melo et al. 1992; Melo et al. 2001). Morphological data and subcellular fractionation experiments suggest that there is a tight association between the PVM and host mitochondria, and that the association is governed by the microtubules (Sinai et al. 1997). Association of host microtubules with *T. parva* schizonts has been described previously (Hulliger et al. 1964; Fawcett et al. 1984) and, in the present study, dual-labelling of TLL with antibodies specific for schizonts and host mitochondria, including hsp60, PDHE1 and ATPase revealed that host mitochondria appeared to be located in the area where schizonts reside (chapter 6). Furthermore, dual-labelling of isolated schizonts revealed a close association of schizonts with host mitochondria (chapter 6). It is therefore possible that host mitochondria might associate indirectly with schizonts via microtubule bridges. However, whether this arangement has a biological purpose, or arises simply because both structures associate with microtubules, remains to be determined.

The microsporidian PV membrane is highly permeable and probably does not pose a significant barrier to solute acquisition by the parasite (Leitch et al. 1995). In

addition, microsporidia have a small genome, apparently due to a loss of many genes encoding proteins in biosynthetic pathways. Also, microsporidian parasites do not have mitochondria (Katinka et al. 2001), and close association with host mitochondria might be beneficial to the parasite in that it relies heavily on the host cell for its energy requirements (Scanlon et al. 2004). In this regard, the identification of four genes for ADP/ATP carrier proteins in the genome of the microsporidia *E. cuniculi* suggests that microsporidia can import host cell ATP (Katinka et al. 2001).

It is interesting to speculate that the close association of *T. lestoquardi* schizonts with host mitochondria reflects a similar metabolic requirement. However, energy metabolism of *Theileria* parasite is unclear, although it has been suggested that the parasite may use glycolysis as their main ATP source (Allsopp et al. 1985). It is generally believed that glycolysis is the main source of ATP in the erythrocytic stages in malaria parasites (Fry et al. 1990). After *P. falciparum* infection, erythrocytes consume up to 100 times more glucose than normal erythrocytes (Pfaller et al. 1982). The TLL multiplication cycle takes 16-20 hrs, as described in chapter 2, and presumably requires considerable energy for proliferation of both the parasite and the host cell. In a study of the metabolic activity of isolated schizonts, Kiama et al. (1999) concluded that enzyme activities were consistent with glucose metabolism being mainly via the Embden-Meyerhof pathway and that the citric acid cycle plays only a minor role. This raises the possibility that *Theileria* parasites also use glycolysis as their main source of ATP.

At present, it is not known whether *Theileria* parasites also use ATP produced by oxidative phosphorylation in mitochondria. In eukaryotic cells, mitochondria are referred as the "powerhouse" as they are the main organelle for ATP production (Garesse et al. 2001). *T. parva* schizonts were observed by Schein et al. (1978) to have normal mitochondrial ultrastructure and to contain clearly defined cristae under the EM. In an attempt to identify drug targets in *Theileria* schizonts, Spooner et al. (1990) indicated that parasite mitochondria protein synthesis was inhibited by oxytetracycline (OTC). OTC mainly inhibits mitochondrial cytochrome c oxidase in mammalian cells (Van Den et al. 1981), and it is used in the "infection and

treatment" method for immunizing cattle against *Theileria* infection (Radley et al. 1975). This suggests that cytochrome c oxidase is active in the parasite mitochondria and that OTC targets enzymes of the respiratory chain.

Some enzymes involved in the citric acid (CTA) cycle were found to be active in isolated *T. parva* schizonts, including malate dehydrogenase, fumarase and succinate dehydrogenase (Kiama et al. 1999). However, other enzymes of the cycle were found to be inactive, including citrate synthase, NADHP+-isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Kiama et al. 1999). The finding suggests that the CTA cycle in the *Theileria* parasite is incomplete. A notable feature of the annotated *T. annulata* genome (http://www.genedb.org/genedb/annulata/index.jsp) is the absence of genes encoding proteins of the mitochondrial complex I site and the F0a and b subunits of complex V (data not shown). This is similar to the situation seen in malaria parasites (Gardner et al. 2002). Thus, it remains unclear whether *Theileria* parasite mitochondria are efficient in producing ATP and the precise metabolic pathways responsible remain to be determined.

Although mitochondria with oxidative phosphorylation activity were found in *T. gondii* parasites, only a single mitochondrion was observed in each tachyzoite (Melo et al. 2000), which suggests that the parasites may not obtain adequate energy from mitochondria. As the distribution of mitochondria within cells can be influenced by localized energy demands (Bereiter-Hahn 1990), the remarkable association of *T. gondii* PVM and host mitochondria (Sinai et al. 1997; 2001) raises the possibility that host mitochondria provide energy for the parasite. There is evidence with some microsporidial infections that host cell oxidative phosphorylation metabolism is significantly increased, implying an increased energy demand on the mitochondria to support parasite proliferation (Weidner et al. 1999). The close association of host mitochondria with the PVM containing *T. gondii* further supports the above hypothesis. Given the incomplete CTA cycle and an apparent absence of genes encoding some of the oxidative phosphorylation subunits in *Theileria* parasites, the close association of host mitochondria with schizonts in TLL might similarly imply

that schizont mitochondria do not contribute sufficient ATP and that schizonts in some way depend on host cell mitochondria for energy.

Unlike *T. gondii* (Mordue et al. 1999) and microsporidia ((Didier et al. 1998), which are bound by the PVM, intracellular *Theileria* schizonts reside free in the cytoplasm (Schein et al. 1978)). This suggests that host nutrients, including ATP products, could more easily gain access to *Theileria* schizonts. *Theileria* schizonts could also more easily recruit host proteins, such as mitochondrial ribosomal protein L12 (MRPL12, TLS4), for their survival (chapter 6). *Theileria* parasites transform host cells and induce uncontrolled proliferation (Fawcett et al. 1982), a unique characteristic compared to other protozoan infections, such as *P. falciparum*, *T. gondii* and microsporidia. The high rate of proliferation of transformed cells requires higher ATP source and, in this regard, the association of host mitochondria with schizonts might have important implications for interactions of the parasite and its host cell.

Future options for the identification of schizont secreted proteins

Improved method for schizont purification

One of the purposes of the present study was to isolate schizonts from infected cells in order to analyse parasite proteins secreted into culture supernatants. A significant contamination of isolated schizonts with host mitochondrial (chapter 2 and 6) resulted in the identification of more host mitochondrial proteins than schizont proteins (chapter 3 and 4). This therefore poses the question of how the method of schizont purification can be improved to yield pure schizonts free from host components.

Because there is a close association of host microtubules with schizonts, therefore, the first consideration would be to disrupt host microtubules. One of the microtubule inhibitors, nocodazole, has been used in purifying schizonts from *T. parva*-infected cells (Baumgartner et al. 1999). Nocodazole belongs to a group of colchicine-site-

binders that interact with tubulin dimers, inhibiting their assembly into microtubules (De Brabander et al. 1976). Adding nocodazole to mammalian cells cultured in vitro results in the loss of most of the cytoplasmatic microtubules including spindles (De Brabander et al. 1976), but, in the case of *Toxoplasma*, has no effect on the parasite (Sinai et al. 1997). Disruption of host microtubules in *T. parva* infected cells using nocodazole has been reported to improve the quantity and quality of purified schizonts (Baumgartner et al. 1999), although this was not corroborated in another laboratory (McKeever D. personal communication) and nocodazole was not incorporated in the protocol used in the present study. However, in light of the problems encountered with mitochondrial contamination, future work in this area should be based on nocodazole treated cells.

As an alternative to using gradients for separating schizonts, the use of magnetized beads coated with a specific antibody for the parasite surface could be considered for schizont purification. It has been reported that magnetic beads coated with an antibody raised against *P. berghei* ookinete surface protein, P28, yield more enriched ookinetes than Nycodenz gradients (Siden-Kiamos et al. 2000). In addition, the magnetic method is quicker, simpler and less expensive than the Nycodenz gradient (Carter et al. 2003; Siden-Kiamos et al. 2000) and it could lead to preparations of purer parasites as well as a higher yield. *Theileria* schizonts could be isolated using magnetic beads coated with an antibody raised against a surface protein, such as IC12.

Selective labelling of parasite proteins

The appearance of labelled host proteins in supernatants of labelled schizont cultures might also be avoided by selective labelling of parasite proteins. This has been achieved using ricin to inhibit host cell protein synthesis in *T. parva*-infected cells, as well as those infected with *T. gondii* and *Eimeria tenella* (Gurnett et al. 1995). Ricin is composed of a glycosidase A chain and a lectin B chain which is necessary for transport across membranes. Once inside the cell, the glycosidase dissociates from the lectin and is released into the cytosol. Without the lectin, the A chain cannot pass through a second membrane, resulting in its exclusion from intracellular parasites

and mitochondria. Therefore, intracellular parasite protein synthesis continues without interruption (Gurnett et al. 1995). However, ricin cannot permeate host cell mitochondria, so that host cell mitochondrial protein synthesis is not inhibited. This can be resolved by the inclusion of the mitochondrial inhibitor chloramphenicol, which does not affect *E. tenella* protein synthesis (Gurnett et al. 1995).

A similar approach might be to isolate schizonts from infected cells and metabolically label them extracellularly. It is not clear whether cultured schizonts would take up sufficient quantities of label and incorporate it into newly synthesised protein. Nonetheless, mitochondria have been isolated from *T. brucei* parasites and metabolically labelled using an established *in organello* system and a number of newly synthesized proteins identified (Hauser et al. 1996; Nabholz et al. 1999). Isolated schizonts were observed to have intact membrane, with membrane potential by staining with MitoTracker dye (chapter 6). Since mitochondria can be isolated from host cells and metabolically labelled, it is possible that schizonts isolated from TLL could be metabolically labelled using the system as described by Nabholz et al. (1999). However, the purity of schizonts, especially those free from host mitochondria would need to be evaluated carefully prior to metabolic label and, if necessary, a host mitochondrial inhibitor should be used.

Bioinformatics

With the availability of the annotated *T. annulata* genome (http://www.genedb.org/genedb/annulata/index.jsp), an obvious option for identification of putative secreted proteins of the schizont is the application of bioinformatics, using algorithms that predict signal sequences.

Appendix I. Buffers and solutions

TLL culture media

Complete Iscove's Modified Dulbecco's Medium (IMDM)

Foetal calf serum (FCS, 5% v/v)	25 ml
L- glutamine (2 mM)	7.30 g
Gentamycin (50 µg/ml)	25 mg
IMDM (Life technologies)	475 ml

Add 25 ml heat inactivated FCS (58 °C for 30 min), L-glutamine, gentamycin to melted IMDM (475 ml) in 37 °C water-bath, mix and keep at 4 °C.

Heparin solution (1000 units/ml)

Heparin sodium salt	100,000 units
PBS	100 ml

Dissolve heparin powder in 100 ml PBS and store at 4 °C.

Concanavalin A (ConA, 1 mg/ml)

ConA	1 mg
PBS	1 ml

Add 1 mg ConA to 1 ml PBS, mix and store in 100 µl aliquots at 4 °C.

Nigrosine solution for cell counting

Stock solution (1%)

Nigrosine	0.1 g
PBS	10 ml

Working solution (0.1%)

1% Nigrosine stock solution	1 ml
PBS	9 ml

Mix and store at 4 °C.

Media for aerolysin production

Davis minimal broth + Yeast extract (DY) medium

K_2HPO_4	7 g
KH_2PO_4	2 g
Sodium Citrate	0.5 g
$MgSO_4.\ 7H_2O$	0.2 g
Yeast extract	10 g
dH_2O	to 1000 ml

Add 900 ml dH_2O, mix, adjust pH to 7.2 using 1M HCl, make up to a final volume of 1000 ml, and autoclave.

10% RNA solution

RNA power	10 g
dH_2O	to 100 ml

Add 80 ml dH_2O, adjust pH to 6.0 using 1M NaOH, and make up to a final volume of 100 ml.

FACS buffer

FCS (5%, v/v)	5 ml
NaN_3 (0.2%, w/v)	0.2 g
PBS	95 ml

Mix and store at 4 °C.

Percoll gradient media buffers

20 × HEPES (pH 7.4)

HEPES (200 mM)	4.76 g
NaCl (3 M)	17.53 g
KCl (400 mM)	2.982 g
dH_2O	to 100 ml

Add 90 ml dH_2O, adjust pH to 7.4 with 1 M NaOH, and make up to a final volume of 100 ml.

1 × HEPES containing 5 mM EDTA (40 ml)

20 × HEPES	2.0 ml
50 mM EDTA	4.0 ml
dH_2O	34 ml

Mix and store at 4 °C.

Stock Percoll (30 ml)

8.5 parts of percoll	25.5 ml
0.5 parts of 20 × HEPES	1.5 ml
1 part 50 mM EDTA	3.0 ml

Mix and store at 4 °C.

Two-dimensional electrophoresis buffers

Lysis buffer

Urea (8M)	19.2 g
CHAPS (4%)	1.6 g
Tris (40mM)	0.194 g
dH_2O	to 40 ml

Prepare and store in 1 ml aliquots at -20 °C.

Rehydration solution with IPG buffer

Urea (8M)	12 g
CHAPS (2%, w/v)	0.5 g
IPG buffer (1%, v/v)	250 µl
Bromophenol blue	a few grains
dH_2O	to 25 ml

Prepare and store in 1 ml aliquots at -20 °C.

SDS equilibration buffer

1.5M Tris-HCl (pH8.8)	6.7 ml
Urea (6M)	72.07 g
87% (v/v) glycerol	69 ml

SDS (2%, w/v)	4.0 g
Bromophenol blue	a few grains
dH_2O	to 200 ml

Prepare and store in 25 ml aliquots at -20 °C.

1.5 M Tris-HCl (pH 8.8)

Tris base (1.5 M,)	181.5 g
dH_2O	to 1000 ml

Dissolve Tris in 900 ml dH_2O, adjust pH to 8.8 with 1M HCl, make up to a final volume of 1000 ml, and store at 4°C.

SDS-PAGE buffer

20% (w/v) SDS solution

SDS (20%, w/v)	40 g
dH_2O	200 ml

Resolving gel buffer (pH 8.8)

Tris (1M)	60.5 g
dH_2O	to 500 ml

Dissolve Tris in 400 ml dH_2O, adjust pH to 8.8 using 1M HCl, and make up to a final volume of 500 ml.

Stacking gel buffer (pH 6.8)

Tris (0.5M)	30.25 g
dH_2O	to 500 ml

Dissolve Tris in 400 ml dH_2O, adjust pH to 6.8 using 1M HCl, and make up to a final volume of 500 ml.

Tank buffer (5×)

Tris (25 mM)	45 g
Glycine (192 mM)	216 g
SDS (20%)	15 ml

dH_2O	to 3000 ml

Dissolve Tris in 2500 ml dH_2O, adjust pH to 8.3 with 1M HCl, and make up to a final volume of 3000 ml.

Laemmmli reducing sample buffer

Stacking buffer (pH 6.8)	2.5 ml
Glycerol	2.0 ml
20% SDS	2.0 ml
β-Mercaptoethanol	1.0 ml
0.1% Bromophenol blue	0.2 ml
dH_2O	to 20 ml

Mix and store at 4°C.

10% (w/v) Ammonium persulphate (APS)

Ammonium persulphate	0.1 g
Distilled water	1 ml

Make fresh before use.

Coomassie Blue staining solution

Coomassie Brilliant blue dye (1%, w/v)	1 g
Glacial acetic acid (10%, v/v)	10 ml
Methanol (50%, v/v)	50 ml
dH_2O	40 ml

Make fresh before use.

Coomassie destain solution

Methanol (25%, v/v)	250 ml
Glacial acetic acid (10%, v/v)	100 ml
dH_2O	650 ml

Make fresh before use.

Coomassie staining solution for PVDF membrane (no acetic acid)

Coomassie Brilliant blue (0.025%, w/v)	0.25 g
Methanol (40%, v/v)	400 ml
dH_2O	600 ml

Make fresh before use.

Coomassie destaining solution for PVDF membrane

Methanol (50%, v/v)	500 ml
dH_2O	500 ml

Make fresh before use.

0.2% Ponceau working solution

Ponceau S concentrate (10%, v/v)	10 ml
dH_2O	90 ml

Make fresh before use.

Immunoblotting solutions

Transfer buffer

Tris (25 mM)	15 g
Glycine (192 mM)	72.06 g
Methanol (25%, v/v)	1250 ml
dH2O	5000 ml

Mix and store at RT.

0.1 M Tris (pH 7.6)

Tris (0.1M)	12.1 g
dH_2O	to 1000 ml

Dissolve Tris in 800 ml dH_2O, adjust pH to 7.6 with 1M HCl, make up to final volume of 1000 ml, and store at RT.

5 mg/ml diaminobenzidine

3,3'-diaminobenzadine	100 mg
0.1 M Tris (pH7.6)	20 ml

Dissolve and immediately dispense into 1 ml aliquots and store at -20°C.

DAB HRP substrate

5 mg/ml diaminobenzidine	1 ml
0.1 M Tris (pH7.6)	19 ml
30%H_2O_2	0.1 ml

Mix and use immediately.

Giemsa staining buffer (pH 7.2)

Na_2HPO4 (7.6 mM)	0.54 g
KH_2PO4 (2.94 mM)	0.2 g
dH_2O	to 500 ml

Dissolve solutes in 400 ml dH_2O, adjust pH to 7.2 with 1M HCl, make up to a final volume of 500 ml and store at RT.

0.4% Trypan blue solution

Trypan blue dye	0.4 g
PBS	100 ml

Dissolve Trypan blue dye in PBS overnight and store at RT.

Formaldehyde fixation solution (3.7%)

37% Formaldehyde	10 ml
PBS	90 ml

Make fresh before use.

Appendix II. PMF and search scores generated by MALDI-TOF analysis

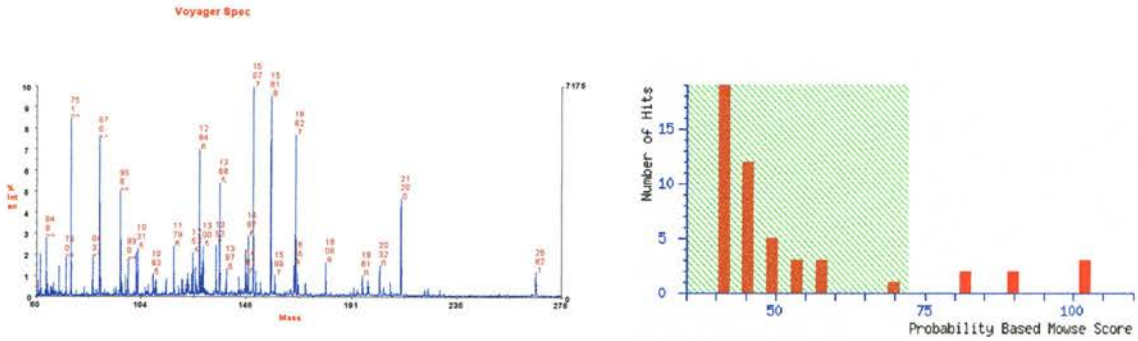


Fig. A-1. TLS1 PMF and search score (significant)
Matched homologous protein, P38646, hsp70, mitochondrial, human.

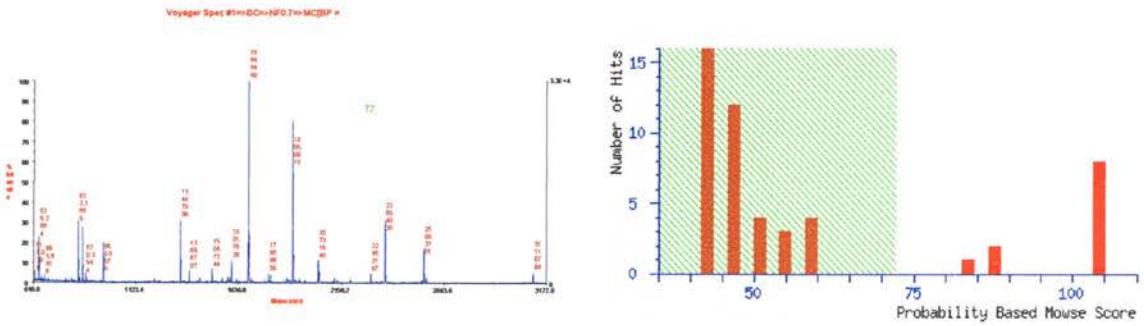


Fig. A-2. TLS2 PMF and search score (significant)
Matched homologous protein, P10809, hsp60, mitochondrial precursor, human.

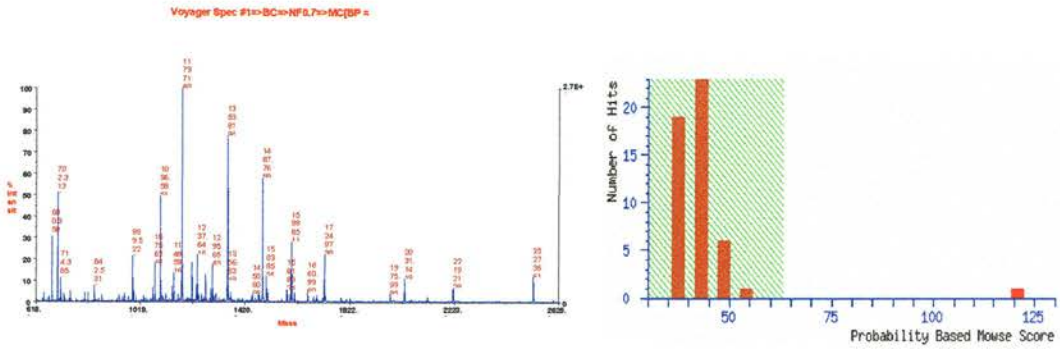


Fig. A-3. TLS15 PMF and search score (significant)
Matched homologous protein, P16019, *Theileria* hsp70.

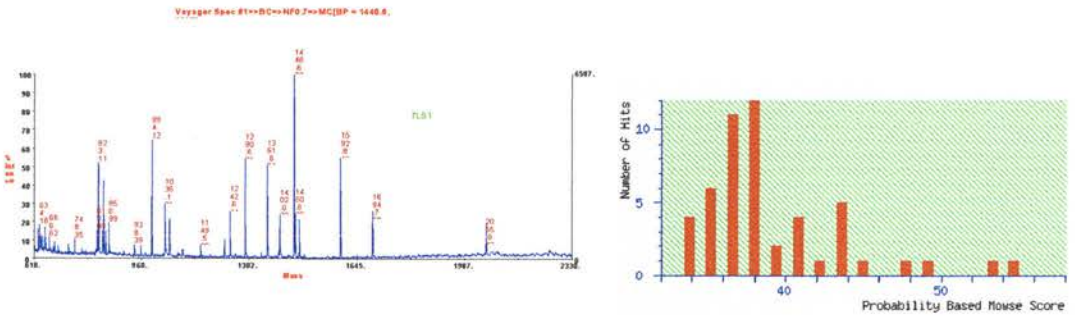


Fig. A-4. TLS3 PMF and search score (insignificant)
Matched homologous protein, AAQ13186, *Theileria* mhsp70.

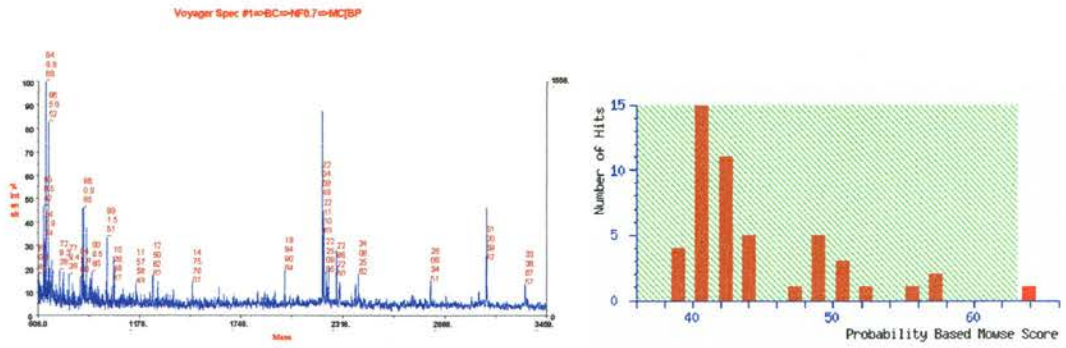


Fig. A-5. TLS4 PMF and search score (insignificant)
Matched homologous protein, NP_081480, mitochondria ribosomal protein L12, bovine.

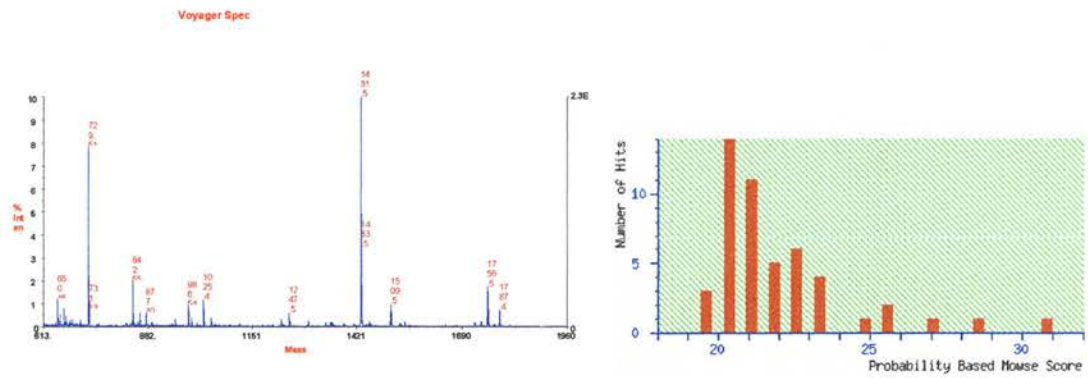


Fig. A-6. TLS5 PMF and search score (insignificant)
Matched homologous protein, P05630, F1-Atpase delta chain, bovine.

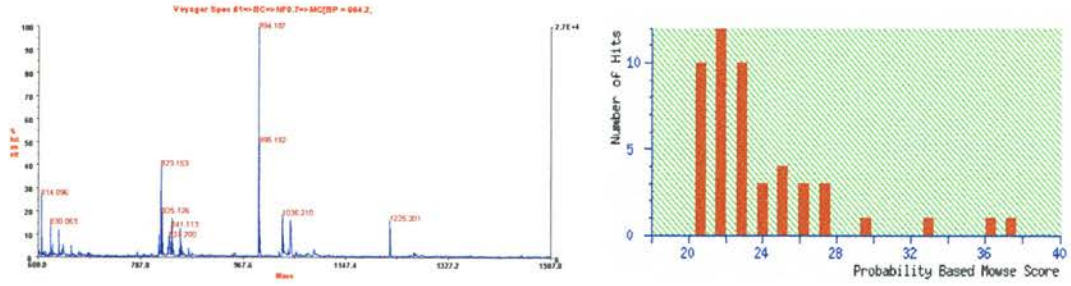


Fig. A-7. TLS11 PMF and search score (insignificant)
Matched homologous protein, P13618, ATP synthase coupling factor 6, bovine.

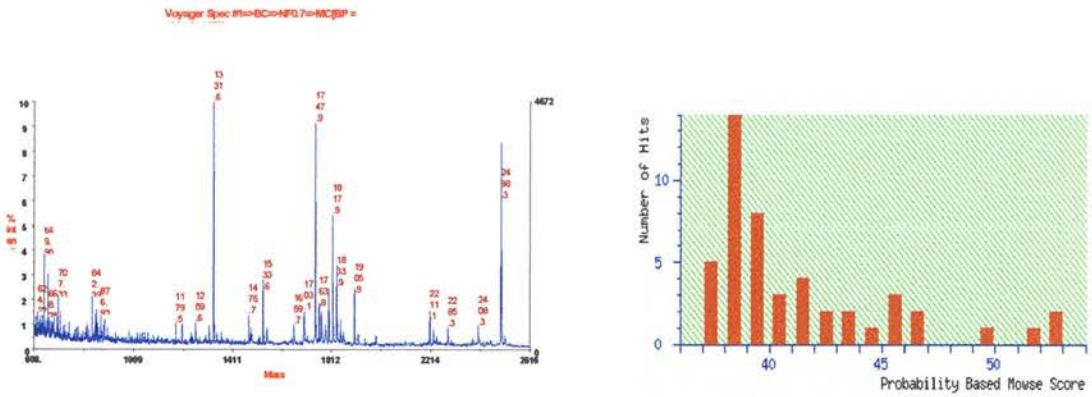


Fig. A-8. TLS12 PMF and search score (insignificant)
Matched homologous protein, gi/1917268B, pyruvate dehydrogenase beta chain, porcine.

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